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## INTRODUCTION

An acknowledged cause of periodontal disease is the dental plaque. The plaque bacteria include oral spirochetes. Treponema denticola and other oral spirochetes may play a significant role in acute necrotizing ulcerative gingivitis and periodontitis (1-3). Thus, a quantitative relationship has been established (4). A recent report (5) indicates that immunosuppressed individuals, such as patients infected with the human immunodeficiency virus have high levels of spirochetes in their oral cavity. However, basic information is needed on the biological attributes of T. denticola that allow it to be a participant in the host-parasite relationship.

The primary aid of the N00014-89-J-3200 (9/89-11/92) grant proposal submitted to the Naval Medical Research and Development Command was to isolate and characterize the outer membrane (outer sheath) of T. denticola. The experiments to be conducted included isolation of outer membranes, determinations of total protein, carbohydrate and ketodeoxyoctonate by chemical analysis, fatty acids by gas chromatography - mass spectral analysis, separation of the solubilized outer membrane proteins by SDS-PAGE, and preliminary tests for the presence of lipopolysaccharide, including limulus amebocyte clotting, chick embryo lethality, mitogenic activity and bone resorption stimulation. During these 3 years of our project progress has been made toward our aim.

Chemical and biological studies on T. denticola outer membrane have been conducted on serovars a, b and c and fresh isolates 7 and 11. Outer membrane potentiates Limulus amebocyte clotting activity and chick embryo lethality. This interesting finding led us to test for additional biological activities that are known to be associated with Gram-negative bacterial endotoxins. In addition, gel electrophoretic patterns, Schiff positive material, and lipid staining material have been reported (6). Quantitative fatty acid analysis by gas chromatography combined with mass spectrometry (GC-MS) has been completed and will be submitted for publication. Recent unpublished studies include the chemical detection of ketodeoxyoctonate, a unique component of endotoxin (lipopolysaccharide, LPS), in outer membrane. Most recently, we have obtained evidence that T. denticola outer membrane contains three biological activities: bone resorption, mitogenic activity and complement activation, all of which suggest the presence of LPS or LPS-like activity in the serovars tested.

To our knowledge this is the first demonstration of LPS biological activity in T. denticola. LPS has been used not only for diagnostic purposes, but it may elicit a wide variety of pathophysiological effects during infection. Further investigations in this laboratory should assist to define the role of LPS in the pathogenicity of oral spirochetes.

## II. DESCRIPTION AND SIGNIFICANCE OF GERMANE RESEARCH FINDINGS

*Paper #1.* *Biochemical properties of the outer membrane of Treponema denticola. J. Clin. Microbiol. 29:1397-1406 (1991).*

The outer membranes (OMs) from serovars a, b, and c of T. denticola, originally isolated from periodontal patients, were prepared. Dialysis of the OMs against 20 mM $MgCl_2$ yielded the aggregable (A) and the nonaggregable (NA) moieties of the OMs. The absence of muramic acid, adenosine triphosphatase, hexokinase, and nucleic acid as well as electron microscopy indicated that the OM preparations were homogeneous. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the A and NA moieties of the OMs showed approximately 25 Coomassie brilliant blue R-250 stain-positive bands or 47 silver-stained polypeptides. The relative molecular masses ranged between 14 and 97 kDa. The electrophoretic polypeptide profiles of the A and NA moieties shared many similarities among serovars a, b, and c. However, they exhibited variation in the overall pattern, intensity, or location of the polypeptide stained zones. This was especially true for serovar b. Two-dimensional electrophoretic studies showed an excess of 100 silver-stained spots with isoelectric points of 4.6 to 7.0 and relative molecular masses in the 14- to 97-kDa range. The OMs contained simple proteins, glycoproteins, and lipoproteins. The NA moieties of the OMs contained 4 to 6, 10 to 12, and 4 to 6 glycopeptides as well as two, seven, and two lipoprotein bands for serovars a, b, and c, respectively. The A moieties of the OMs showed 7 to 9, 11 to 13 and 5 to 6 glycopeptides as well as four, five, and three lipoprotein bands for serovars a, b, and c, respectively.

**LPS staining.** Pronase or proteinase K digestion of the NA moieties of serovars a, b, and c yielded about two, six, and five LPS bands, respectively, with relative molecular masses of 15 to 66 kDa. The 66- and 53-kDa bands were present in serovars b and c but were absent in serovar a, while a band with an approximate relative molecular mass of 45 kDa was present in serovar c but not in serovars a or b. The 66- and 53-kDa bands of serovar b were more prominent than those for serovar c were. The 66- and 53-kDa LPS bands were found in the three serovars of the A moieties, but were so faint that they could not be photographed. Several bands with relative molecular masses of 10 to 15 kDa were very prominent in the A moieties of the three serovars, and they appeared very faint or absent in the NA portion of the outer membranes of serovars a, b, and c.

To improve the resolution of the LPS bands described above, hot phenol extraction of the LPS and SDS-PAGE experiments were conducted. The NA moiety of serovar b had a distinct band with a relative molecular mass of 24 kDa that was absent from serovars a and c. The 66- and 53-kDa bands were found in both the NA and A moieties of serovars a, b, and c as well as the E. *coli* LPS, which was used as a control. It should be pointed out that the hot phenol LPS extraction procedure eliminated the 10-kDa band found in LPS preparations involving the use of proteolytic enzymes.

**Limulus assays.** Gelation of the *Limulus* amebocyte lysate has been used as a sensitive means for the detection of endotoxin. Since the electrophoretic studies indicated that LPS was present in the A and NA moieties of the OMs of *T. denticola*, *Limulus* tests were conducted to obtain additional evidence for the presence of endotoxin in the OM of *T. denticola*. Table 1 summarizes the results of these experiments. It is apparent that both the A and NA moieties of the OMs of serovars a, b, and c showed *Limulus* amebocyte lysate clotting activity. The A moieties had gelation endpoints in the 5-ng range, while the NA moieties, with the exception of serovar b, had gelation endpoints of 5 to 15 ng. The *E. Coli* endotoxin control had a gelation endpoint of 0.5 to 1 ng.

TABLE 1. *Limulus* amebocyte lysate clotting activity of the outer membrane of *T. denticola*

| Sample concn (ng) | *Limulus* lysate clotting activity[a] | | | | | |
|---|---|---|---|---|---|---|
| | Serovar a | | Serovar b | | Serovar c | |
| | A moiety | NA moiety | A moiety | NA moiety | A moiety | NA moiety |
| 225 | + | + | + | + | + | + |
| 113 | + | + | + | + | + | + |
| 52 | + | + | + | + | + | + |
| 26 | + | + | + | + | + | + |
| 11 | + | - | + | + | + | - |
| 5.5 | + | - | + | + | + | - |
| 2.8 | - | - | - | + | - | - |
| 1.4 | - | - | - | - | - | - |
| 0.7 | - | - | - | - | - | - |
| 0 | - | - | - | - | - | - |

[a]Data were obtained from seven determinations.

[b]+, solid clot in the bottom of the tube; -, no clot.

**Chick embryo lethality assays.** We conducted chick embryo assays to extend our work on the LPS found in the OMs of *T. denticola*. The data obtained from these assays are given in Table 2. With the exception of serovar c, doses of 48 to 80 μg of the NA moieties and 200 to over 400 μg of the A moieties of the OMs of *T. denticola* were required to kills the 11-day-old chick embryos. *E. coli* LPS had a lethal dose close to that found for the A moieties of serovars a and b.

TABLE 2. Chick embryo lethality of the outer membrane of *T. denticola*

| Material inoculated[a] No. inoculated | 100% mortality | Cocn ($\mu$g) yielding |
|---|---|---|
| A moiety, serovar a | 27 | 210-315 |
| NA moiety, serovar a | 22 | 68-80 |
| A moiety, serovar b | 28 | 316-407 |
| NA moiety, serovar b | 25 | 48-60 |
| A moiety, serovar c | 23 | >400 |
| NA moiety, serovar c | 21 | 300-409 |
| *E. coli* LPS | 19 | 50-100 |
| Control (no OM) | 17 | No deaths |

[a]The indicated material was diluted in pyrogen-free water and was inoculated on the chorioallantoic membrane of 11-day-old chick embryos.

The detection of LPS-like components in the outer membrane of *T. denticola* is an interesting finding brought out by this study. Lipopolysaccharides (endotoxins) have been used for diagnostic purposes and they may elicit a wide variety of pathophysiological effects. However, presently the role of LPS in spirochetes remains unknown and further investigations will be required to define its role in *T. denticola*, or other oral spirochetes.

***Paper #2.*** *Effect of outer membrane of Treponema denticola on bone resorption. Oral Microbiol. and Immunol. (accepted for publication 11/30/92).*

Bone resorption represents a well recognized problem in periodontitis, and endotoxins (LPSs) are generally considered participants in bone resorption (7). Results in our laboratory using 2-keto-3-deoxyoctonate analysis as an estimate of the presence of presumed lipopolysaccharide (LPS) in T. denticola outer membrane indicated that LPS might be present at the level of 0.5 - 1.3% by weight in T. denticola whole bacteria. Pregnant Sprague-Dawley rats were injected subcutaneously with 200 $\mu Ci^{45}$ Ca (specific activity 10-40 mCi/mg Ca) on the 18th day of gestation. One day later, the rats were sacrificed, the fetal radii and ulnae were removed by microdissection, and explanted as pairs for organ culture in BGJ medium. Following addition of 5% fetal bovine serum, 50 units/ml penicillin, 50 $\mu$g/ml streptomycin and 500 ng/ml fungizone to the BGJ culture medium, the bones were set up in sterile culture. Cultures consisted of 2 bones (opposite limbs from the same fetus), which were incubated at 37°C for 24 h in a 5% $CO_2$ incubator in the presence or absence of varying concentrations of outer membrane of T. denticola. Bones heated at 75±5°C for 4 min prior to culture in the BGJ organ medium were used as negative controls. Six to eight pairs of bones were used for each experiment.

Bone resorption was assessed by the release of previously incorporated $^{45}Ca$ from the shafts of the radii and ulnae of 19 day fetal rats cultured in BGJ medium. The ratio of the release of previously incorporated $^{45}Ca$ from bones treated (T) with outer membrane to that released from

paired control (C) bones was used to measure bone resorption. A mean T/C ratio significantly greater than one, indicated stimulation of bone resorption by the test substance. Statistical significance was assessed by the paired student "t" test. Addition of the outer membrane of T. denticola added to calcium labeled embryonic radii and ulnae increased $^{45}Ca$ release from the cultured bones.

Figure 1 shows the results for serovar b. A sham outer membrane preparation served as a negative control. Error bars represent standard error of the mean (SEM). The minimum concentrations of the outer membranes of serovars a, b and c of T. denticola required to yield significant $^{45}Ca$ release were those containing 15, 22 and 75 $\mu$g protein respectively. Heating the T. denticola outer membrane at 60°C for 30 min prior to addition to bone cultures, did not change the effect on $^{45}Ca$ release. Typically T/C ratios of 1.2-1.4$\pm$0.04 were obtained. This finding suggests that the bone resorption stimulating factor present in the outer membrane of T. denticola was heat stable, as would be expected of LPS.

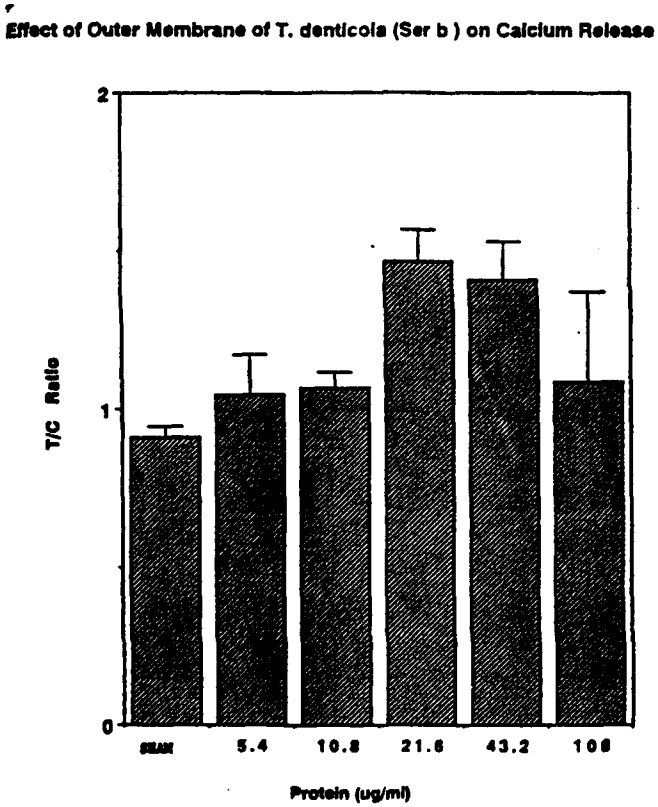


Fig. 1

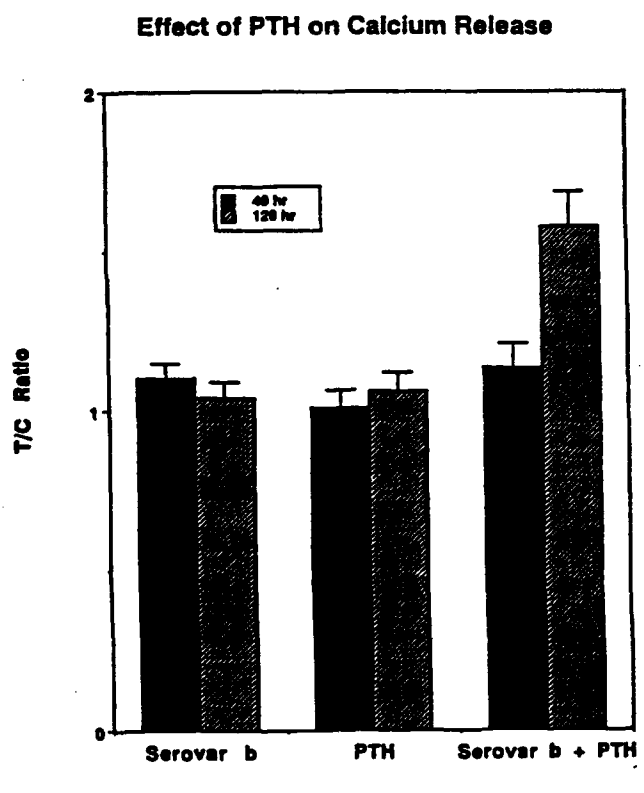


Fig. 2

Purified LPS (5$\mu$g) of E. Coli and Salmonella typhimurium yielded T/C ratios of 1.35 $\pm$ 0.11 and 1.48 $\pm$ 0.08, respectively. On the basis of this finding, there would be about 1 to 5 $\mu$g of E. coli-type LPS in the outer membranes of serovars a, b and c that contained 15, 22 and 75 $\mu$g protein. Roughly 2 to 3 $\mu$g of LPS as estimated by KDO analysis would accompany these

amounts of protein. KDO is a unique constituent of many LPSs, but not all. The assumption is also made that KDO represents 1% of the weight of the LPS molecules, a very rough assumption considering the variable nature of LPSs. We do not know yet whether the LPSs from T. denticola serovars are of the smooth, rough, or of an intermediate type. It is also worthy of note that estimates of LPS content from KDO analysis obtained for T. denticola serovar b (strain 33521) contained about two times more LPS in its outer membrane than serovars a or c.

Synergistic action of T. denticola outer membrane with prostaglandin $E_2$ and with parathyroid hormone in bone resorption. To strengthen the suggestion that the active component in the outer membrane of T. denticola is an LPS, the effect of parathyroid hormone and of prostaglandin $E_2$, both of which are known to act synergistically with LPS in bone resorption (8), was tested.

It was found that the outer membrane of T. denticola serovar b at a concentration of 10 $\mu$g protein per ml and lower yielded the same $^{45}Ca$ release from the rat bones as the controls. That is, the T/C ratios were 1.100 ± 0.049 and 1.040 ± 0.051 at 48 and 120 h, respectively. Parathyroid hormone at a concentration of 40 ng per ml also produced no significant stimulation in the bone resorption assay system. However, when the outer membrane of T. denticola serovar b and parathyroid hormone were added together to the bone resorption assay system at these levels, $^{45}Ca$ release from the bones was enhanced significantly at 120 h of incubation, but not at 48 h, yielding T/C ratios of 1.580 ± 0.108, and 1.135 ± 0.077, respectively (Fig. 2). This synergistic activity with parathyroid hormone and $PGE_2$ in bone resorption is similar to that described for known purified endotoxins (55).

Effect of prostaglandin $E_2$ on $^{45}Ca$ release. It has been well established that prostaglandin $E_2$ is a potent stimulator of bone resorption in organ culture. In our assay system we used $PgE_2$ at suboptimal concentration and studied its effect on $^{45}Ca$ release with or without the outer membrane. Addition of $PGE_2$ at very low levels ($10^{-7}$M) did not stimulate $^{45}Ca$ release from Ca labeled fetal rat bones. However, when the bones were exposed to outer membrane of serovar b at 10 $\mu$g protein/ml, as above, together with $PGE_2$ at $10^{-7}$M, there was significant $^{45}Ca$ release at 120 h (T/C ratio = 1.31 ± 0.073).

**Paper #3.** *Fatty acid profiles of the outer membrane of ATCC strains 35405, 35404 and 33521 of Treponema denticola. J. Periodont. Res. (Submitted for publication.) Also abstract #1 J. Dent. Res. 70 (spec. issue): 580 (1990).*

The fatty acid composition of the outer membrane (outer sheath) of Treponema denticola is not known. Furthermore, fatty acids are constituents of lipopolysaccharides. This study examined the fatty acid profiles of the outer membranes of T. denticola ATCC strains 35405, 35404 and 33521. Homogeneous outer membranes were prepared from the three strains of T. denticola. The fatty acids were extracted and converted to methyl esters, and their mass spectra were determined with a sensitive Hewlett-Packard 5880A-5970 gas chromatography-mass spectrometry system. Fatty acids were identified by comparing the unknown fatty acid mass

spectra to computer stored known mass spectra standards. Dodecanoic, 2 hydroxy dodecanoic, tridecanoic, tetradecanoic, pentadecanoic, hexadecanoic, 2-hydroxy hexadecanoic and octadecanoic acid were found in the assay spirochetes yielding correlation indices (r) of 0.8-1.00. Isotetradecanoic acid was found in the outer membranes of strains 33521 and 35405 (r=0.913-0.967). Anteiso pentadecanoic and heptadecanoic acids were found in the outer membrane of strains 33521 and 35404 (r=0.941-0.996), while cis 9, 12 octadecadienoic was found only in the outer membrane of strain 35405 (r=0.922-0.958). The average concentration of dodecanoic, tridecanoic, tetradecanoic, pentadecanoic, hexadecanoic, heptadecanoic and octadecanoic acid in the outer membranes of strains 35405, 35404 and 33521 were as follows. Strain 35405: 138, 178, 845, 296, 751, not detected, and 699 nanogram per mg dry weight of the outer membrane. Strain 35404: 96, 125, 670, 306, 597, 38, and 249 nanograms per mg dry weight of the outer membrane. Strain 33521: 323, 135, 1650, 125, 9080, 235 and 618 nanograms per mg dry weight of the outer membrane.

This is the first report on the fatty acid profiles of the outer membrane of strains 35405, 35404 and 33521 of T. denticola. The experiments described here are in harmony with our previously reported data which suggested that the outer membrane of T. denticola contains lipoproteins and lipopolysaccharide. Furthermore, this study indicates that fatty acid analysis may be used as an additional tool for the characterization of strains 35405, 35404 and 33521 of T. denticola.

*Abstract #2. Mitogenic activity in outer membrane of Treponema denticola. 1992. J. Dent. Res. 71 (Sp. Issue):318.*

Previous studies in our laboratory using the limulus lysate clotting and chick embryo lethality assays demonstrated an endotoxin like activity in outer membrane (OM) of *T. denticola*. To extend these findings, mitogenic and KDO assays were conducted.

Mitogenic stimulation of C3H/HeOuJ spleen cells. Measurements of mitogenic activity were made using murine spleen cells from endotoxin sensitive 8 to 12 week old mice (C3H/HeOuJ) which were cultured in microliter 96-well plates at 2x $10^6$ cells/ml for 44 h at 37°C in a 5% $CO_2$ incubator. The medium used was RPM1-1640 fortified with L-glutamate, vitamins, penicillin, streptomycin, gentamicin, amphotericin B, essential and non-essential amino acids, 1% mouse serum, sodium pyruvate, 2-mercaptoethanol, hepes buffer. NaOH was used to adjust the pH to 7.2. All operations were carried out under sterile conditions. Each assay was set up in triplicate with four-fold dilutions of each serovar outer membrane. The outer membrane material was vortexed 2 to 3 min at 24°C, prior to pipetting in the same manner that one would handle E. coli LPS. The mitogenic activity was quantified by adding a pulse of 1$\mu$Ci per well [$^3$H-methyl] thymidine at the 44th hour of incubation and allowing the incubation to continue an additional 6 hours. Cells were harvested onto Xtalscint glass fiber filters using a PhD harvester, dried overnight and counted in a scintillation counter. E. coli 055:B5 LPS (Sigma) was used as a standard positive control. A sham preparation of outer membrane was used as a negative control. In some cases polymyxin B at a concentration of 0.01 mg/ml was added to the cells 30 minutes prior to the addition of the outer membrane in order to test whether it would prevent the mitogenic activity, as it does against E. coli LPS.

Mitogenic activity was tested with the outer membrane isolated from T. denticola serovars a (ATCC 35405), b (ATCC 33521) and c (ATCC 35404) as well as from fresh isolates 7 and 11. Stimulation of $^{3}$H-thymidine uptake in mouse spleen cell culture was directly related to the concentration of outer membrane, both from the ATCC serovars (Fig. 3) and from the fresh isolates (Fig. 4). A sham outer membrane preparation gave no mitogenic stimulation (Fig. 4). Polymyxin B sulfate at 0.01 mg/ml, added 30 min prior to addition of the outer membrane dilutions, inhibited mitogenic stimulation by 10-20% in all the serovars. The same concentration of polymyxin B was much more effective against a purified preparation of E. coli 055:B5 LPS (Fig. 5). Similar results were obtained when polymyxin B was added to outer membrane of another spirochete, B. burgdorferi, by another group. Polymyxin B interacts with the E. Coli lipid A moiety of LPSs of enterobacteria. Perhaps there is a different type of lipid in spirochetal LPS or perhaps other macromolecules in this crude outer membrane preparation mask the LPS. Studies are planned to isolate T. denticola LPS in our laboratory and retest purified material. From the KDO analysis, about 1$\mu$g of LPS is associated with 10 $\mu$ of outer membrane protein. This means that significant mitogenic stimulation (four times control level or above) occurs between 0.5 and 5 $\mu$g of presumed T. denticola LPS. Significant stimulation was also observed with 0.2 to 5 $\mu$g E. coli purified LPS.

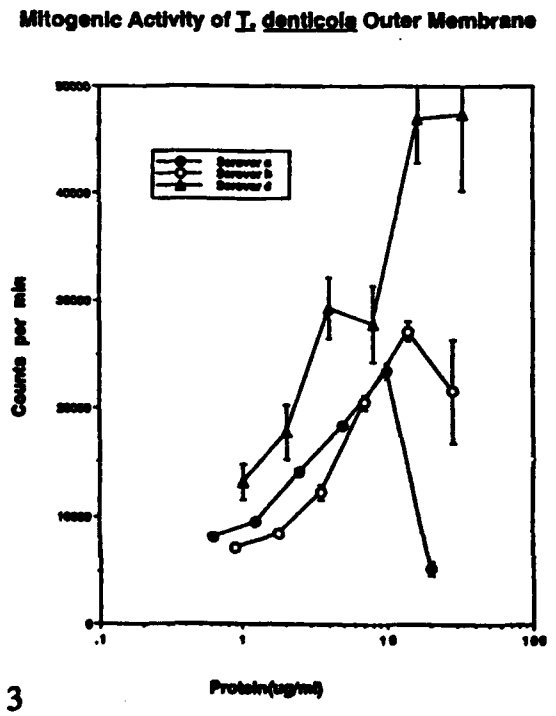


Fig. 3

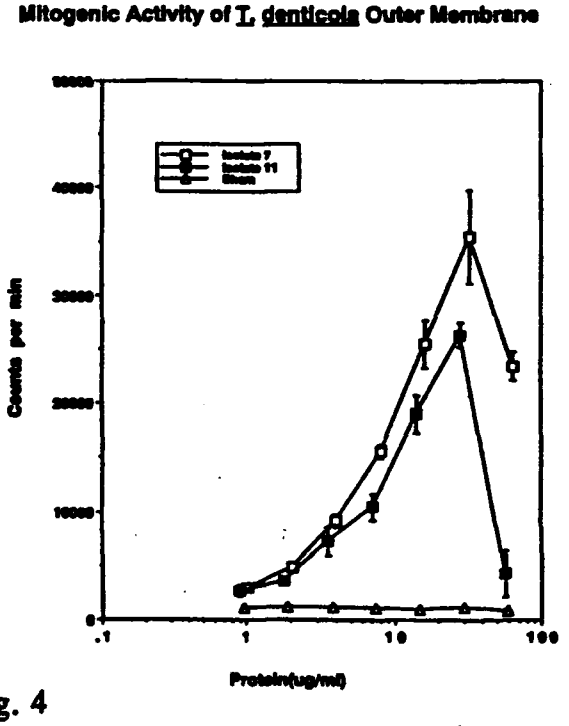


Fig. 4

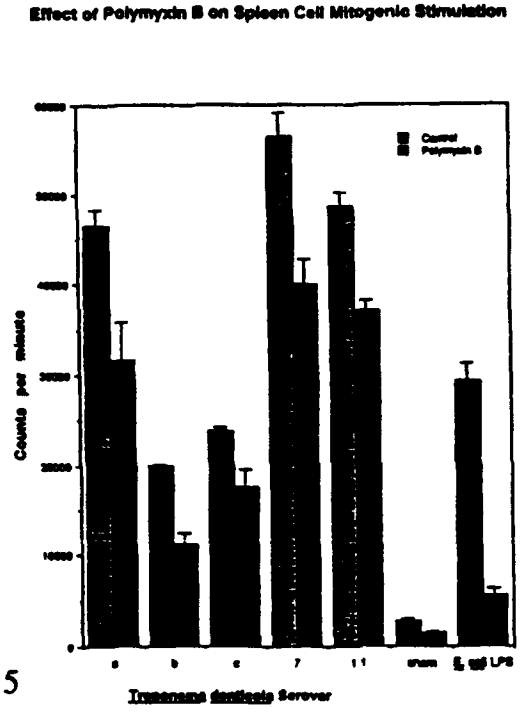


Fig. 5

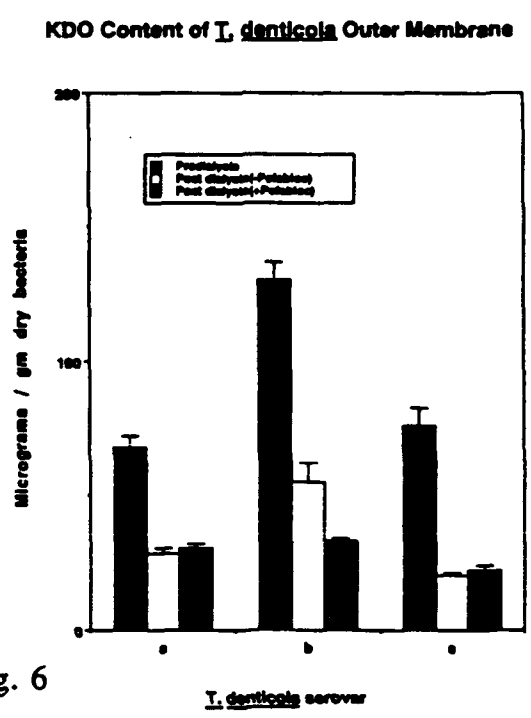


Fig. 6

Ketodeoxyoctonate. 2-Keto-3-deoxyoctonate (KDO), a unique component of LPS was measured. Four separate undialyzed preparations of the outer membranes of serovars a, b and c and a sham control of T. denticola were analyzed for their KDO content. The outer membranes (outer sheath) of T. denticola serovars a, b and c were extracted with 0.7 mM SDS. Whole extracts in the presence of the SDS were digested for 1 hour at 100°C in 0.1 M acetate, pH 4.4 according to Brade, et al (9). S. typhimurium LPS (Sigma) was used as a positive control to check on the digesting procedure. Purified KDO was used to standardize the assay with thiobarbituric acid. The presence of 0.7 mM SDS and 2 x $10^{-4}$M Pefabloc (a protease inhibitor) did not interfere with the assay. The 500 $\mu$l digested material containing 400 $\mu$l sample and 100 $\mu$l 0.5 M acetate, pH 4.4 was centrifuged to remove the precipitate, and 250 $\mu$l was removed for the KDO assay. Then 10 $\mu$moles of $HIO_4$ and 50 $\mu$moles of $NaASO_2$ per tube was added. S. typhimurium LPS (100$\mu$g) after acid digestion produced 0.98% by dry weight) KDO. This is the expected amount, if the LPS contains about 2% KDO, since the 0.1 M acetate, pH 4.4 hydrolysis yields only 1/2, or 2/3, of the actual amount of KDO depending on whether 2 or 3 KDO residues are present on the LPS. Only the outer 1 or 2 residues of KDO are assayed and not the KDO residue attached to the lipid A moiety of the LPS (9).

Various LPS molecules that have been studied so far contain a total of 2 to 3 KDO molecules each. The total amounts of KDO, in $\mu$g per g dry weight of cells, found in the outer membrane extract of serovars a, b and c of T. denticola, were 64.3 ± 2.7 (S.E.M.), 106.8 ± 14.0, and 71.3 ± 7.5, respectively. From these values, if one makes the assumption that the same proportion of KDO is present in LPSs of T. denticola as is present in S. typhimurium LPS, then the quantity of LPS in T. denticola is estimated to be 0.6%, 1.1% and 0.7%, for serovars a, b and c, respectively. The quantity of KDO in the dialyzed outer membrane preparations was approximately half that estimated for the whole outer membrane prior to dialysis, probably due to adherence of LPS to the dialysis tubing during concentration. Low molecular weight cut-off

tubing (2,000 Mr) was used to prevent losses through the tubing. The KDO value for b post dialysis (+ Pefabloc) is probably low due to mechanical loss noted during dialysis changes. It is interesting to note that serovar b contains a higher quantity of LPS in the outer membrane, which may in part explain the observation that serovar b is considered somewhat more pathogenic than a and c (4). Lipopolysaccharide may contribute to the local inflammation observed in periodontal pockets.

Complement activation. Schenkein and Berry (10) reported activation of complement by whole bacteria of T. denticola FM and TD2. Kearns, et al (11) typed FM as serovar b and TD2 as non-a, non-b and non-c. In collaboration with Dr. Gregory Spear at Rush Presbyterian-St. Luke's Hospital, Chicago, we have now shown that complement activation for ATCC 33521 (also serovar b) resides in the outer membrane preparation. Outer membrane from serovars a and c did not activate complement until 100-fold higher concentration was reach. A sham control had no effect (Fig. 7).

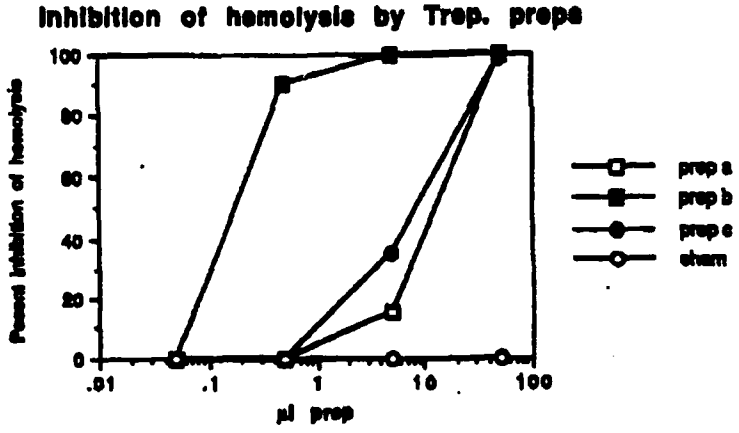


Fig. 7. Inhibition of hemolysis by outer membrane of T. denticola.

Lipopolysaccharides (LPSs) can initiate activation of serum complement depending upon the structure and chain length of the polysaccharide attached. Inhibition of lysis of sensitized sheep erythrocytes was measured. Samples of dialyzed outer membrane from serovars a, b, c and a sham preparation in 100 µl RPMI-1640 medium were incubated with 550 µl of a 1:12 dilution of normal young rabbit serum (the complement source) at 37°C for 30 min. Then 400 µl of sensitized sheep red blood cells (5 x $10^8$/ml) was added and incubation was continued for an additional 30 min. Cells were diluted with 2 ml of ice cold saline containing 0.01 M EDTA to stop the reaction and centrifuged. The absorbance of supernatants was measured at 412 nm and the percent hemolysis was calculated relative to the hemolysis of controls in which medium was added with no outer membrane preparation. Curves were produced using varying amounts of dialyzed outer membrane and a determination of the 50% lysis point was read from the graph. Lysis inhibition of 50% was obtained with 20 µl, 0.2 µl and 10 µl that contained 30µg, 0.2µg, and 7µg outer membrane protein for serovars a, b and c, respectively (Fig. 7). Quantities of 0.1, 1, 10, and 100 µl of a sham preparation gave no inhibition of lysis. It required 4 µg of

purified S. typhimurium LPS to give 50% inhibition of complement mediated lysis. These experiments need to be repeated to verify the results. If the quantities stated are confirmed, it would appear that substances other than LPS, such as proteases, may be responsible for inhibition of lysis.

Inflammatory events associated with periodontitis may be related to and exacerbated by the complement system. Plasma proteins and complement components have been shown to be present in gingival fluid. Bacteria such as T. denticola may activate the complement system resulting in the binding of various complement proteins, or their cleavage products, to specific receptors on neutrophils, macrophages, platelets, mast cells and erythrocytes. This in turn triggers phenomena such as phagocytosis, alteration of vascular permeability, cytolysis, lymphokine production, lysosomal enzyme release and bone resorption (12).

*Abstract #3. Human antibodies to Treponema denticola outer membrane. 1993. J. Dent. Res. 72 (Sp. issue).*

Western immunoblotting with human sera. Electrophoresis was carried out for the first step for immunoblotting and then the antigens transferred onto charged nylon membranes. Human antisera were diluted 1:50 and incubated overnight with blotted outer membrane proteins prepared from T. denticola serovars a, b and c and from fresh isolates 7 and 11. Goat anti-human IgG(H+L chains) conjugated with alkaline phosphatase was used as secondary antibody. Color was developed for the same length of time in all cases.

Western blots of three periodontitis sera are shown in Fig. 8 (Panels B, C and D). The periodontitis sera all showed strong reactivity with antigens other than those seen in the control serum shown in Panel A. Of six control sera, five were obtained from adults with healthy gingiva and one from a healthy 3-year old. Of these six control sera, the three-year old and one adult showed only three faint bands seen in Panel A (Fig. 8). Two of the other four controls exhibited three more faint bands and two exhibited patterns which were similar in some respects to patterns with sera from periodontitis patients. In four of the five Western blots using the periodontitis sera, the three faint bands seen in this control were also visible (Fig. 8, Panels B and D). Sera from five adults with severe periodontitis showed strong antibody reactivity both to the different serovars and to very different molecular weight antigens in these serovars.

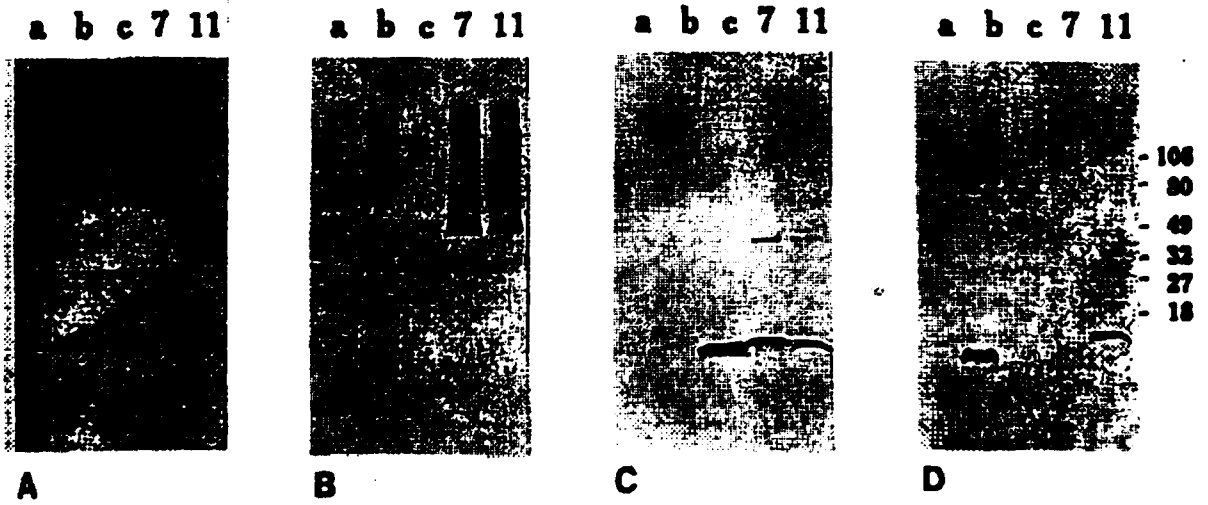


Fig. 8

Fig. 8. Western immunoblots of human sera from patients with periodontitis. The molecular weights of the prestained standards provided by the manufacturer are given on the verical axis (Fig. 8). From comparisons with wide range standards made on Coomassie blue stained gels, it appears that values for these prestained standards ought to be revised upward about 2 kDa.

Sera from periodontitis patients had marked reactivity to one or two bands of high molecular weight material (>40 kDa) and/or to bands of low molecular weight (<15 kDa). Serum from patient (B) reacted to 44 kDa material in outer membrane from isolates 7 and 11. Serum from patient (D) reacted to a 8-10 KD fragment in serovar b, and to a 12 kDa fragment in isolate 11.

Another patient's serum (not shown) exhibited a pattern similar to patient B's with additional antibody directed against serovar a at 10 kDa, 35 kDa and 80 kDa. The Western blot of the serum from the fifth patient resembled D.

Sera tended to show a smear when they reacted with the 44 kDa band of isolates 7 and 11. If the 44 kDa band was dark, the lane contained a smear in the area of higher molecular weight material above the 44 kDa band. This indicates that there is either higher molecular weight material which is immunoreactive, or that the 44 kDa material is sparingly soluble in the SDS buffer used for gel electrophoresis. The alkaline phosphatase immunoblotting is a sensitive technique and will pick-up immunoreactive material even in very low concentrations trailing behind. However, it is also possible that the smear effect is due to LPS, which by the nature of its varying number of repeating sets of sugar units, is known to show a ladder-like pattern, often a smear in gel electrophoresis (13). LPSs from smooth types of Gram-negative organisms tend to form a ladder which extends to much higher molecular weights. LPSs from rough and semi-rough types form a single band or a closely spaced ladder at low molecular weights (13). Another possibility is that the smear might be due to antibody reactivity against a sparingly soluble lipoprotein, as is the case with an immunodominant lipoprotein of T. pallidum (6).

There appear to be three bands which react to a lesser extent in 10 of the 11 human sera tested. These are a doublet of bands at 30 and 31 kDa present in all serovar outer membrane preparations and a high molecular weight smear (120-200 kDa) in outer membrane of T. denticola serovar b. Whether the doublet corresponds to the 31.5 kDa and 35.8 kDa T. denticola axial filament proteins reported by Nilius and Simonson (14), we do not know yet. Serum from two controls reacted only to these "common" antigens, but to no others (Fig. 8, Panel A). Rabbit polyclonal antibody obtained from Dr. L. Simonson and made against formalized whole T. denticola serovar a, b or c, produced 10-20 bands per lane on Western blots. Notably, the patterns using polyclonal rabbit antiserum made against ATCC 35405 and ATCC 35404 appeared quite similar to each other but dissimilar to ATCC 33521.

A sham outer membrane preparation, prepared at the same time as the outer membrane preparations from the serovars, showed no bands at all when gels were stained with Coomassie blue for protein or with the more sensitive silver staining technique, and it did not react at all in Western blots. Transblotted serovar preparations to which no primary antibody (human sera) was added showed no reactivity. Backgrounds were clear and bands were usually quite distinct.

Admittedly, this is a very small sampling of sera from patients with periodontitis, but the results already show several different immune responses to the different strains of T. denticola. Studies to date using human sera against T. denticola antigens have mainly used enzyme linked immunosorbent assays (ELISAs) (4,15). Only three previous studies reported on periodontitis sera which reacted in Western blots. In one study nine periodontitis sera were reacted against a single T. denticola serovar, ATCC 33520 (serovar c). A 53 kDa major reactive antigen was observed when either rabbit polyclonal sera or the periodontitis sera were tested against whole cell lysates (19). In a second study, four patient sera were also reacted against T. denticola ATCC 33520 as well as against three fresh isolates. In the third study, patient serum was used to show that a cloned 53 kDa protein corresponded to an immunogenic protein.

Califano (16) reported that the immunodominant antigens to A. actinomycetemcomitans did not always produce strong bands on Western blots at low dilution of antibody. Therefore, in order to titer the antibody to find the most immunodominant antigens, we will have to make serial two or four fold dilutions of the sera and test the dilutions against the panel of antigens. The highest dilution at which there is still a band(s) remaining on a Western blot will define the most immunodominant antigen(s).

Our techniques for performing Western blots differed from those published for T. denticola (17-19) in several major ways. We used 4-20% gradient mini gels, instead of standard 10% or 12% polyacrylamide gels. Lower molecular weight polypeptides become visible on gradient gels, whereas they diffuse or even run off standard gels. We used charged nylon membranes and fixed the electroblotted proteins with glutaraldehyde, a suggestion from Dr. L. Simonson's laboratory. This seems to work well, although there is a possibility that some antigenic determinants could be destroyed by the procedure, since glutaraldehyde covalently links proteins and other molecules to the nylon through their -$NH_2$ and -COOH groups. The alkaline-phosphatase conjugated secondary antibody provides a more sensitive method than the horseradish peroxidase and is not inhibited by azide, if azide is used as a preservative in handling samples. The assay can be made even more sensitive by increasing the time of color development. Our standard incubation time was forty minutes, although prominent bands showed up in five minutes.

*Abstract #4. Human antibodies to Treponema denticola match marine monoclonals. 1993. J. Dent. Res. 71 (Sp. issue).*

Recently, we found that there was a strong correlation between the presence of ADA Type III and IV periodontitis and antibody reactivity to low apparent molecular weight components (Mr < 18kDa) of outer membrane isolated from T. denticola. There was a more pronounced

antibody response and a response to a greater number of low Mr constituents of the T. denticola serovars in the severe periodontitis groups compared to controls. Monoclonal antibodies (mAbs) were selected from murine antibodies against T. denticola whole cells to correspond to four serovars, a, b, c and d. All these mAbs also reacted with low Mr components in isolated outer membrane preparations. We then determined whether the human patient sera reacted with the same antigens as the mAbs. The sera from three patients were tested in side-by-side comparisons at 1:50 dilution with mAbs using immunoblotting techniques. One patient serum (#12) reacted with strong bands to a ~ 12 kDa component in the outer membrane of serovar b and a ~ 15 kDa component from isolate 11 (serovar d). The human antibody(ies) matched the mAb to b and mAb to d. Another serum (#8) reacted with a ~ 16 kDa component from serovar a (ATCC 35405) only and this band matched the mAb to a. However, a third serum (#10), which reacted with what appeared to be the same two low Mr components to which the first serum reacted showed that the patient serum and mAb against b were reacting with two different components. These patient sera and mAbs may be useful reagents for the characterization of human antibody response to T. denticola in periodontitis.

*Paper #4.* *A study of the acid phosphatase of Treponema denticola. 1990. Zbl. Bakt. 274: 195-202.*

*Abstract #5.* *Isolation and partial characterization of acid phosphatase of Treponema denticola 1990. J. Dent. Res. 69 (Sp. issue): 183*

*Abstract #6.* *Phosphorylated nutrient uptake by potential periodontopathogens. 1991. J. Dent. Res. 70 (Sp. issue): 462.*

Research Aim 2: Utilization of Phosphorylated Nutrients by Periodontopathogens. The second specific aim of our research plan was to assay potential periodontopathogens for their ability to concentrate radiolabeled, phosphorylated key nutrients following dephosphorylation by periplasmic phosphatases presumed to be present in the periplasmic space of T. denticola (20).

2a. A study on acid phosphatase of T. denticola. Further efforts were made to conclude prior investigations and provide additional evidence for the periplasmic location of acid phosphatase. A paper has been submitted and accepted for publication (21). This manuscript describes some of the properties of the potential periodontopathogen T. denticola serotype c (ATCC strain 33520). The highest enzyme activity was found in 87 h old cells. Two optimum pHs for enzyme activity were detected, one at pH 4.8 and another at pH 6.2. Divalent cations did not influence the acid phosphatase of T. denticola. The anion $F^-$ added in the form of NaF and at a level greater than 20 $\mu$g/ml $F^-$ diminished the activity of the acid phosphatase of intact cells of T. denticola. The addition of 10 $\mu$g/ml $F^-$ as $SnF_2$ induced statistically significant reduction of acid phosphatase activity.

The apparent $K_m$ for the acid phosphatase was 7.3 mM with p-nitrophenyl phosphate as substrate. Fluoride appeared to be a noncompetitive inhibitor of the enzyme with an apparent $K_i$ of 0.3 mM. Acid phosphate may be released partially in osmotic shock fluids. Also, 7-

diazonium-1, 3-naphthalene disulfonate, which is incapable of penetrating the bacterial permeability barrier and is known to inactivate enzymes found in the bacterial periplasmic space, suppressed the activity of the acid phosphatase in intact cells of T. denticola.

The acid phosphatase could be released partially from the serotype c by the osmotic shock procedure (22) and a 20-fold purification of acid phosphatase has been achieved by DEAE-cellulose chromatography (23).

The enzyme degraded a variety of phosphate containing substrates including p-nitrophenol phosphate, adenosine triphosphate, glucose-1-phosphate, fructose-1, 6-diphosphate and inorganic pyrophosphate. The addition of 40-100 $\mu$g/ml $F^-$ induced a statistically significant reduction of acid phosphatase.

2b. Studies on the Uptake of Selected Phosphorylated Nutrients by Periodontopathogens. The three serotypes a, b and c of T. denticola were grown anaerobically in the GM-1 medium for 5-6 days. For the fast grown anaerobes Actinobacillus actinomycetemcomitans ATCC 29522, Bacteroides gingivalis ATCC 33277 and Fusobacterium nucleatum ATCC 25586 3 day old cultures were employed. The assay cells were collected by centrifugation and washed twice with deionized double distilled water. A homogeneous suspension was prepared by aspiration through a syringe with a 25-27 gauge needle. The suspensions were then adjusted to an optical density of 2.0 at 650 nm. The bacterial suspensions were incubated for 1 h in the anaerobic incubator to suppress the endogenous reserves of nutrients, and aliquots of the suspensions were used for the uptake experiments. The uptake was initiated by the addition of 0.5 ml of the bacterial suspension to 0.5 ml of the assay mixture containing 0.01 $\mu$Ci [U-$^{14}$C] adenosine-5' - triphosphate, $\alpha$-D-[U-$^{14}$C] glucose-1-phosphate, or D-[U-$^{14}$C] fructose-1, 6 diphosphate. Following incubation for varying time intervals, the cell pellets were washed twice with distilled water or acetate buffer. The cells were solubilized with 10% SDS and the radioactivity found in the solubilized cells was used to determine the uptake of radiolabeled, phosphorylated nutrients. Oral spirochetes heated at 65°C for 1 h were used as controls.

2c. Optimization of the Uptake of Phosphorylated Nutrients and Evaluation of Plaque Parameters Affecting Nutrient Uptake. These experiments were performed to systematically derive the operational conditions of phosphorylated nutrient uptake required for optimal response, as well as reproducibility, and to assess the dental plaque conditions that may influence nutrient uptake.

2d. Selection of Cell Concentration: Since the amount of phosphorylated nutrient accumulated by the bacterial cells may be small and only an adequate cell concentration can yield reliable, reproducible nutrient uptake, attempts were made to establish the optimal cell mass for the nutrient uptake studies. In general, washed cell suspensions of the assay cells at 0-5 mg, or more than 5 mg dry weight, were incubated anaerobically with sufficient levels of the radiolabeled, phosphorylated nutrient. Then following incubation the uptake of the assay nutrient was determined by the general procedure. There was a linear relationship of the uptake of radiolabeled ATP of glucose-1-phosphate with cell concentrations in the range of 0.1-

2.5 mg of cell dry weight. Above the 2-3 mg cell concentration level the uptake curve of the phosphorylated assay nutrients reached a plateau. Thus, in all of the subsequent uptake experiments 2-3 mg of cell dry weight were employed for the general assay system.

2e. Estimation of the End-point of Uptake. Meaningful and useful comparisons of uptake experiments of phosphorylated nutrients by test microorganisms can only be made with uptake systems that have attained equilibrium. Time course studies were conducted to determine the time interval required to attain the end point of the uptake of the assay nutrients. Thus, accumulation of the phosphorylated compounds by the test cells were allowed to proceed for varying time intervals and the time required for the uptake experiments to reach equilibrium were determined by the general assay procedure. The time course of the uptake of α-D-[U-$^{14}$C] glucose-1-$PO_4$, or [U-$^{14}$C] adenosine-5' -triphosphate was determined. The uptake of the radiolabeled assay nutrients reached a maximum value within 2-5 min and remained constant for the next 55 min. Therefore, for the ensuing uptake experiments a period of 30 min was used routinely. The percent uptake was nearly 3 times higher (27%) when the cells were washed with water, than with 0.2 M acetate buffer, pH 4.8. Hence water washed cells were used in all of the uptake experiments.

2f. Nutrient Uptake and Temperature. Conditions of growth in broth, saliva, dental plaque, and subgingival crevice where potential periodontopathogens may be found differ. Thus, the uptake of phosphorylated nutrients by the assay cells could be influenced by changes of temperature in the oral cavity, or subgingival crevice. The accumulation of phosphorylated, radiolabeled ATP and glucose-1-phosphate was assessed by the usual general uptake procedure, but under varying temperatures. At temperatures of 4-30°C the uptake ranged between 13-29%. At temperatures 50°C the assay cells took up 0-1% of the test nutrients.

2g. pH and Nutrient Uptake. The pH of the periodontal pocket varies with respect to pH. Therefore, it was pertinent to assess the uptake of radio-labeled assay nutrients under diverse hydrogen ion concentrations. It was found that uptake of [U-$^{14}$C] adenosine triphosphate was maximum at pH 4.8. α-D-[U-$^{14}$C] glucose-1-$PO_4$ uptake by the assay cells showed two pH maxima. The nutrient uptake maximum peaks for serotypes a and c occurred at pH 4.8 and 8.9, while for serotype b, which is thought to be more pathogenic than serotypes a or c, occurred at pH 5.8 and 7.8.

2h. Antiplaque Agents and Phosphorylated Nutrient Uptake. A number of antiplaque agents have been shown to decrease the number of subgingival motile bacteria, or the bleeding index score (24). The mechanism of action of these antiplaque remain uncertain, and it argues for further investigation in this area. To determine if inhibition of the uptake of the phosphorylated nutrients is occurring by interference with the dephosphorylating action of the phosphatases of the assay spirochetes, the following experiments were performed. Under optimal conditions established previously, cell suspensions of the assay organisms were incubated and processed as usual. However, the antiplaque agents were added to the assay system.

The five commercially available antiplaque agents known as Plax (Oral Research Laboratories), Cepacol (Lakeside Pharmaceuticals), Viadent (Viadent Co.), Listerine Antiseptic (Warner-Lampert Co.), and Fluorigard (Colgate-Palmolive Co.), at a dilution of 1:10 induced a 50-90, 50-70, 50-64, 50-56 and 8-41% inhibition respectively in the uptake of [U-$^{14}$C] adenosine triphosphate by the three serotypes. Stannous fluoride at a concentration of 10 $\mu$g/ml enhanced the uptake of radiolabeled ATP, or glucose-1-$PO_4$, indicating possible damage of the spirochetal membrane.

2i. Comparative Uptake of Phosphorylated Nutrients by Potential Periodontopathogens. A. actinomycetemcomitans, P. gingivalis and F. nucleatum, which have been implicated in the pathogenesis of periodontitis possess acid and alkaline phosphatase (20). Phosphatase could be involved in the dephosphorylation and subsequent uptake of various nutrients. Thus, the comparative accumulation of [U-$^{14}$C] adenosine triphosphate and $\alpha$-D[U-$^{14}$C] glucose-1-phosphate by A. actinomycetemcomitans, P. gingivalis, F. nucleatum and T. denticola was determined by the general uptake assay procedure at a pH of 4.8 and 6.5. The uptake of phosphorylated ATP by A. actinomycetcomitans, P. gingivalis, F. nucleatum and T, denticola at pH 4.8 was 13, 1.5, 6.9 and 20.4% respectively. At a pH of 6.5 the percent uptake for the microorganisms indicated above was 22, 1.3, 6.6 and 6.2 respectively. The accumulation of radiolabeled, phosphorylated glucose-1-$PO_4$ at pH 4.8 was 4.2, 0.6, 0.7, 35.4% for A. actinomycetemcomitans, P. gingivalis, F. nucleatum and T. denticola respectively. At a pH of 6.5 the levels of accumulation of glucose-1-$PO_4$ by the assay cells changed to 16.7, 0.9, 4.3 and 4.9% respectively.

During tissue destruction phosphorylated nutrients are released from oral tissues. There are fluctuations in the number and species of these plaque flora during health and dental disease. The factors governing these microbial changes in the dental plaque are not known. Nutrient uptake and utilization is thought to play a role in the observed bacterial ecology (25). This hypothesis is supported by the data obtained from this study. Furthermore, this study provides specific examples of phosphorylated nutrient accumulation by potential periodontopathogens. Such examples were lacking (26).

The metabolic versatility of T. denticola appears to contribute to its survival in the competitive environment of the gingival sulcus (26). The experiments on the uptake of radiolabeled, phosphorylated ATP and glucose-1-$PO_4$ tend to support the view that there are variations in the accumulation, or hydrolysis of useful phosphorylated nutrients by T. denticola, A. actinomycetemcomitans, P. gingivalis and F. nucleatum. These differences in nutrient uptake may contribute to the survival of T. denticola in the competitive environment of the gingival sulcus. The utilization of nucleotides such as ATP could involve prior dephosphorylation by periplasmic phosphatases. The periplasmic phosphatases act as scavenging enzymes, hydrolysing nontransportable, phosphorylated nutrients into components that can then be transported and utilized by microorganisms (23).

Limited information is available on the acid phosphatase of potential periodontopathogens. Initial studies in our laboratory indicate that T. denticola, A. actinomycetemcomitans, P.

gingivalis and F. nucleatum possess acid phosphatase. However, while a 45-60 min incubation period was required to demonstrate this enzyme in T. denticola the other assay periodontopathogens required 180-240 min of incubation with p-nitrophenol phosphate (20) as substrate. Extension and confirmation of related work showed that useful substrates such ATP, glucose-1-phosphate, or other nutrients were hydrolyzed by a phosphatase that could be released from the periplasmic space of T. denticola, and be purified by DEAE column chromatography. Thus, the periplasmic acid phosphatase of T. denticola can act as a scavenging enzyme degrading nontransportable, phosphorylated useful nutrients that can then be taken up and utilized by this oral spirochete.

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## IV. PUBLICATIONS

(A). Full length published papers (Reprints enclosed)

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2. Yotis, W.W., Sharma, V.J. Gopalsami, C., Chegini, s., McNulty, J., Hoerman, K., Keene, J., and Simonson, L.G. 1991. Biochemical properties of the outer membrane of Treponema denticola. J. Clin. Microbiol. 29:1397-1406.

(B). Full length paper accepted for publication (copy enclosed)

1. Gopalsami, C., Yotis, W., Corrigan, K., Schade, S., Keene, J. and Simonson, L. 1993. Effect of outer membrane of Treponema denticola on bone resorption. Oral Microbiol. and Immunol.

(C). Full paper submitted for publication.

1. Yotis, W., Keene, J., Hoerman, K. and Simonson, L.G. 1992. Fatty acid profiles of the outer membrane of ATCC strains 35405, 35404 and 33521 of Treponema denticola. J. Periodont. Res.

(D). Published Abstracts

1. Yotis, W., Sharma, V.K., Hoerman, K., Keene, J. and Simonson, L.G. 1990. Outer Sheath polypeptides of Treponema denticola. J. Dent. Res. 69 (Sp. issue): 183.

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# Biochemical Properties of the Outer Membrane of *Treponema denticola*

WILLIAM W. YOTIS,[1]* VIJAY K. SHARMA,[1] CHELLAM GOPALSAMI,[1] SIMA CHEGINI,[1] JOHN McNULTY,[2] KIRK HOERMAN,[3] JOSEPH KEENE, JR.,[3] AND LLOYD G. SIMONSON[4]

*Departments of Microbiology,[1] Anatomy,[2] and Periodontics,[3] Loyola University Medical Center, Maywood, Illinois 60153, and Naval Dental Research Institute, Great Lakes, Illinois 60088-5259[4]*

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**The outer membranes (OMs) from serovars a, b, and c of *Treponema denticola*, originally isolated from periodontal patients, were prepared. Dialysis of the OMs against 20 mM $MgCl_2$ yielded the aggregable (A) and the nonaggregable (NA) moieties of the OMs. The absence of muramic acid, adenosine triphosphatase, hexokinase, and nucleic acid as well as electron microscopy indicated that the OM preparations were homogeneous. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the A and NA moieties of the OMs showed approximately 25 Coomassie brilliant blue R-250 stain-positive bands or 47 silver-stained polypeptides. The relative molecular masses ranged between 14 and 97 kDa. The electrophoretic polypeptide profiles of the A and NA moieties shared many similarities among serovars a, b, and c. However, they exhibited variation in the overall pattern, intensity, or location of the polypeptide stained zones. This was especially true for serovar b. Two-dimensional electrophoretic studies showed an excess of 100 silver-stained spots with isoelectric points of 4.6 to 7.0 and relative molecular masses in the 14- to 97-kDa range. The OMs contained simple proteins, glycoproteins, and lipoproteins. The NA moieties of the OMs contained 4 to 6, 10 to 12, and 4 to 6 glycopeptides as well as two, seven, and two lipoprotein bands for serovars a, b, and c, respectively. The A moieties of the OMs showed 7 to 9, 11 to 13 and 5 to 6 glycopeptides as well as four, five, and three lipoprotein bands for serovars a, b, and c, respectively. Lipopolysaccharide was detected in the OMs of the three serovars following removal of proteins with proteinase K, pronase and silver staining of sodium dodecyl sulfate-polyacrylamide gels, or removal of lipopolysaccharide from the OMs by hot phenol extraction. The 66- and 53-kDa bands were present in serovars b and c, while a band with a relative molecular mass of 45 kDa was present only in serovar c. Endotoxin-like activity was also shown in the OMs of the three serovars by the *Limulus* amebocyte clotting assay and the chick embryo lethality test. This is the first report on selected biochemical properties of the OM macromolecules of three known serovars of *T. denticola*.**

An acknowledged cause of periodontal disease is the bacteria found in the dental plaque (26). Intermediate-size oral spirochetes, including *Treponema denticola*, have been associated with acute necrotizing ulcerative gingivitis (27, 31). The three serovars a, b, and c of *T. denticola* have been described previously (7, 34). Using monoclonal antibodies developed against serovar "b," Simonson et al. (33) provided evidence of a positive relationship between *T. denticola* serovar "b" and severe periodontitis. This spirochete was present at a significantly elevated level in plaque samples collected from deep subgingival pocket sites of patients with severe periodontitis (33). *T. denticola* produces immunosuppressive substances (3), tissue-damaging enzymes (30), and metabolic products and antigens which are potentially harmful (27).

The structure of *T. denticola* is, in general, similar (5) to that of the other spirochetes and consists of a typical cytoplasmic membrane composed of phospholipids and proteins or enzymes such as adenosine triphosphatase, periplasmic flagella, a cell wall containing muramic acid, and an outer membrane (OM), or outer sheath, which can be removed by treatment with 1.4 mM sodium dodecyl sulfate (20). Limited information is available on the macromolecular composition and function of the OM of oral spirochetes. For example, by using intact cells, cell lysates, and immunoelectron microscopy, a 53-kDa antigen has been found in the outer membrane of *T. denticola* serovar c (34, 40). In the present investigation, the macromolecular profiles and some biological properties of clean OM preparations of *T. denticola* serovars a, b, and c are presented.

## MATERIALS AND METHODS

**Isolation of the OM.** *T. denticola* ATCC 35405 (serovar a), ATCC 33521 (serovar b), and ATCC 35404 (serovar c) were used in this study. These strains were originally isolated from human periodontal pockets. The spirochetes were cultured anaerobically at 35°C for 5 to 8 days in the prereduced medium of Blakemore and Canale-Parola (2) which was supplemented with 5% (vol/vol) heat-inactivated rabbit serum. Cells from 15 liters of cultures of the assay treponemes in the late logarithmic phase of growth were removed by centrifugation at 12,000 × *g* for 30 min, washed two times with deionized doubly distilled water, and lyophilized. The OMs were removed from the spirochetes by treatment for 15 min with 1.4 mM sodium dodecyl sulfate (SDS) and subsequently isolated as described by Johnson et al. (20). The adopted protocol involved the following modifications. Phenylmethylsulfonyl fluoride (1 ml of a 100 mM solution) was added to the OM isolation mixture to prevent protein degradation by serine proteases. Furthermore, fol-

* Corresponding author.

lowing the 15-min exposure of the spirochetes to 1.4 mM SDS and the removal of cells by centrifugation, the supernatant was passed through a 0.22-μm-pore-size filter instead of a 0.45-μm-pore-size Millipore filter. This was done to avoid contamination of OMs by intact spirochetes, which can pass through 0.45-μm-pore-size filters. Dialysis of the outer membranes against 20 mM $MgCl_2$ yielded the aggregable (A) and the nonaggregable (NA) moieties of the OM. $MgCl_2$ treatment was designed for the enrichment of the OM macromolecules. In contrast to the previous isolations of spirochetal OMs (20), in this study the OMs were assayed for possible contamination by using DNA, RNA, and hexokinase as markers of cytoplasmic constituents; ATPase as a cytoplasmic membrane marker; and muramic acid as a peptidoglycan marker (4, 6, 11, 12, 21, 23). Furthermore, the morphology of the OMs and the spirochetes from which the OMs were removed was determined by electron microscopy (20). Briefly, preparations containing 100 to 300 μg of the A and NA moieties of the OMs were used for biochemical assays. DNA was determined by the diphenylamine method, using calf thymus DNA as the standard (4). RNA was determined by the method of Griswold et al. (11), using yeast RNA (type III; Sigma Chemical Co., St. Louis, Mo.) as a standard. Agarose gel electrophoresis was also used to assay for DNA (19). Hexokinase was measured by an NADP-linked assay (21), which measured the increase in NADPH $A_{340}$. The assay mixture contained 0.1 ml of phosphate buffer (0.1 M, pH 7.5), 0.2 ml of 0.15 M glucose, 40 μl of 1 M $MgCl_2$, 100 μl of 13 mM NADP, 2 U of glucose 6-phosphate dehydrogenase, 100 to 300 μg of the NA or A moiety of the OM, and 81 mM ATP; and the volume was brought to 2 ml with deionized distilled water. Baker's yeast hexokinase (sulfate-free; EC 2.7.1.1; type F 300; Sigma) was used as a standard. Controls consisted of reaction mixtures in which glucose 6-phosphate dehydrogenase, the source of hexokinase, or glucose was omitted. ATPase was measured by the liberation of $P_i$ (6, 23) in 1 ml of reaction mixture containing 2.5 mM ATP (vanadate-free), 100 mM KCl, 4 mM $MgCl_2$, and 50 mM Tris-acetate buffer (pH 6.5). The reaction was started by the addition of the NA or A moiety of the OM containing 100 to 300 μg of protein, and it was terminated by the addition of 0.5 ml of 1.5 M perchloric acid. The controls included the reaction mixture to which the sample was added after perchloric acid treatment. Muramic acid was determined by the colorimetric *p*-hydroxybiphenyl method of Hadzija (12). Positive controls included mixtures to which known quantities of muramic acid were added. Negative controls included mixtures without the recommended reagents. The muramic acid used as a standard was treated identically as the NA and A moieties of the OMs were.

Protein estimations of samples containing mercaptoethanol were performed by the protocol of Hill and Straka (13), which uses complex formation of bicinchoninic acid with copper and iodoacetamide binding of free mercaptoethanol. The A moieties were solubilized with EDTA at a final concentration of 5 to 8 mM. The OM moieties that did not contain mercaptoethanol were processed directly with bicinchoninic acid reagent (35).

**Electron microscopy.** Spirochetal preparations were fixed with 1% $OsO_4$, dehydrated, and embedded in Epon. Sections were cut on an LKB III ultratome and collected on Parlodion-covered copper grids. The sections were stained with 2% uranyl acetate and examined with an Hitachi HV-IIB electron microscope operated at 75 kV (28).

**Electrophoretic studies.** SDS-polyacrylamide gel electrophoresis (PAGE) was done on 12.5% (wt/vol) polyacrylamide gels essentially by the method of Laemmli (24). However, 0.8% piperazine diacrylamide (Bio-Rad, Richmond, Calif.) replaced bisacrylamide to reduce background staining, increase physical gel strength, and reduce gel swelling (15, 16). Charcoal treatment of acrylamide and piperazine diacrylamide was also used to remove some impurities in the gels. Finally, SDS was omitted from the separating gel, but the concentration of SDS was increased to 0.2% in the running gel buffer to reduce interference by SDS during treatment with the silver staining reagents (15, 16, 29, 37). SDS-PAGE was performed at 4 to 6°C in a Hoeffer SE 600 gel chamber with a current of 15 mA per gel until the tracking dye reached the separating gel, and then a constant current of 30 mA per gel was used for approximately 4 h. Generally, the following proteins (Bio-Rad and Sigma) were subjected to electrophoresis as described above for SDS-PAGE and served as relative molecular weight standards: β-galactosidase, 116,250; phosphorylase *b*, 97,400; bovine serum albumin, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin soybean inhibitor, 20,100; and lactalbumin, 14,200. Gels were stained with 0.2% (wt/vol) Coomassie brilliant blue R-250 in 50% ethanol–5% acetic acid or with an ammoniacal silver nitrate solution (1, 16, 17).

**Periodic acid-Schiff staining in SDS-polyacrylamide gels.** Following electrophoresis, the gels were processed as described by Fairbanks et al. (8) for glycoprotein detection. Samples to be compared for glycoprotein staining were electrophoresed in the same slab so that a side-by-side comparison could be made of the protein components. A known glycoprotein control was electrophoresed and processed simultaneously. The periodate step was omitted as an additional control. The Schiff reagent was freshly prepared.

**Apolipoprotein staining.** Detection of apolipoprotein was made by comparison of the lipase-treated and untreated samples following SDS-PAGE. To samples containing 150 μg of protein, 8.5 U of yeast lipase type VII (EC 3.1.1.3; Sigma) was added to a reaction mixture consisting of the sample, lipase, 50 mM Tris hydrochloride (pH 7.4), and 10 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 2 h. Then, usually 30 ml of 5× Laemmli sample loading gel buffer was added, and the samples were processed for SDS-PAGE (24). Beta-serum lipoprotein fraction III (National Biochemical Products, Cleveland, Ohio) was used as an apolipoprotein standard. Following SDS-PAGE, the gels were placed for 24 h in a solution composed of 0.4% Sudan black dissolved in ethylene glycol–15% acetone–12% glacial acetic acid. The gels were destained with the solution described above, but without the Sudan black dye (10).

**Detection of LPS.** Two approaches were used to detect lipopolysaccharide (LPS) in the A and NA moieties of the OM. The first method consisted of pronase or proteinase K digestion of the OM moieties prior to SDS-PAGE, periodate oxidation of LPS, and silver staining of the gels (30). Briefly, samples containing 100 μg of protein were placed in the sample lysing buffer and prepared for SDS-PAGE (24). For protein digestion, 0.25 μg of proteinase K per μg of the sample dissolved in the lysing buffer was added and incubated at 60°C (14). Following SDS-PAGE, the gels were fixed in a solution containing 40% ethanol and 5% acetic acid. LPS oxidation was then attempted for 10 min with 0.7% periodic acid dissolved in a mixture composed of 40% ethanol–5% acetic acid (39). The gels were washed three times with deionized water for 20 min each time and stained with silver nitrate. The second method for LPS detection

was a slight modification of the procedure described by Kido et al. (22), in that a hot phenol extraction (65°C for 10 min) instead of the cold phenol treatment was used to extract the LPS from the OMs. *Escherichia coli* endotoxin O55:B5 (Sigma), untreated or treated, was used as a standard. Two-dimensional PAGE was performed as described previously (16, 30).

***Limulus* test.** Experiments designed to detect LPS in the A and NA moieties of the OMs were conducted by the method of Levin and Bang (25). New disposable plasticware found to be free of endotoxin was usually used. Sterile and pyrogen-free glassware, when needed, was prepared by autoclaving it for 45 min, followed by heating it for 3 h at 175°C in a dry oven. All tests were conducted in new disposable polystyrene culture tubes (10 by 75 mm), which were immediately capped after the reaction mixtures were added. All endotoxin-free reagents required for the performance of the *Limulus* test, which was supplied in a kit with assay instructions known as E-Toxate (*Limulus* amebocyte lysate), were purchased from Sigma. Optional endotoxin-free reagents, such as 0.1 N HCl and 0.1 N NaOH for pH adjustment of the samples to be tested at the optimum range of pH 6.8 to 7.5, were also purchased from Sigma. E-Toxate had a sensitivity of 0.025 to 0.1 endotoxin unit per ml toward the standard *E. coli* O55:B5 LPS. Dilutions of test and control samples (*E. coli* LPS in pyrogen-free water) were incubated in tubes with amebocyte lysate at 37°C for 1 h, and then the tubes were inverted. Any tube which did not contain a hard gel that did not adhere to the bottom of the tube was scored as negative.

**Chick embryo lethality.** Large white eggs obtained from a single flock of Illinois hens were used in all experiments. The eggs were incubated at 37.0 ± 0.5°C and rotated daily. On day 11 of incubation, the eggs were candled, the chorioallantoic membrane was dropped, and the substance to be tested was inoculated onto the choriallantoic membrane (36). The eggs were broken 18 to 24 h later and examined for dead embryos. As was the case with the *Limulus* assays, only sterile pyrogen-free plasticware and glassware were used during the chick embryo assay. Dilutions of test and control samples were made in pyrogen-free water (Sigma). Bacterial contamination of the eggs was rare, and experiments in which it occurred were discarded. The experiments described here were repeated three to seven times and were reproducible, and the typical data depicted in Fig. 1 to 7 or Tables 1 to 3 were obtained.

## RESULTS

**Release of the OM.** Electron microscopy of serovars a, b, or c indicated that exposure of *T. denticola* serovar a, b, or c to 1.4 mM SDS for 15 min removed the outer membranes of the assay spirochetes without disturbing the typical protoplasmic cylinder. The electron micrographs indicate that the periplasmic flagella remained attached to protoplasmic cylinders. Others may have been removed with the protoplasmic cylinders during the separation of the OMs from the spirochetes. Also, electron microscopy of the A and NA moieties of the OM preparations did not show any periplasmic flagella (Fig. 1). Similarly, biochemical analysis for DNA, RNA hexokinase, ATPase, and muramic acid indicated that neither the A nor the NA moiety of the OM contained any detectable cytoplasmic contents, cell membrane, or peptidoglycan markers (Table 1). The yield of the OMs ranged between 4 and 7% of the cell dry weight and was approximately equally divided between the A and NA moieties.

**OM polypeptide characterization by SDS-PAGE.** The NA moieties of the OMs of *T. denticola* serovars a, b, and c showed 10, 8, and 12 Coomassie brilliant blue R-250-stained polypeptides, respectively. Upon SDS-PAGE, the A moieties revealed 15 to 22 Coomassie brilliant blue R-250-stained polypeptides for serovars a, b, and c. The majority of the polypeptides had a relative molecular mass that ranged between 14 and 97 kDa. Some faint bands had a relative molecular mass of 10 kDa. The electrophoretic polypeptide profiles of both moieties of the three serovars showed many similarities. A band with a relative molecular mass of approximately 50 kDa was found in both the A and NA moieties of the three serovars. However, upon close examination, they revealed slight variations. For example, the A moiety of serovar b had more bands near the 35- to 66-kDa range than serovars a or c did, and it also formed a band at the 20-kDa-molecular-mass scale (Fig. 2).

Detection of the OM polypeptides was greatly improved by silver staining of the SDS-polyacrylamide gels. A protein load of 15 μg resolved approximately 47 silver-stained polypeptides for both the A and NA moieties of the OMs of serovars a, b, and c. The relative molecular masses ranged between 14 and 97 kDa. The electrophoretic polypeptide profiles of the A and NA moieties remained constant, and they shared many similarities, forming common bands at the 50- to 53-kDa-molecular-mass range. However, they exhibited variation in the overall pattern, intensity, or location of the stained zones of polypeptides. For example, the A moiety of serovar a showed a well-spaced ladder-like electrophoretic profile and contained fewer bands than the A moieties of serovars b or c did. Furthermore, a 17-kDa band present in the NA moieties of the three serovars was absent in the A moieties of serovars a, b, and c (Fig. 3).

**Glycoprotein staining.** An effort was made to assess the OMs for the presence of glycoproteins. Therefore, following SDS-PAGE of the protein samples of the OM proteins, the gels were processed for periodic acid-Schiff staining of glycopolypeptides (8). The experimental protocol revealed 7 to 9, 11 to 13, and 5 to 6 stained bands of glycopolypeptides for the A moiety of the serovars a, b, and c, respectively. The NA moieties of the OMs of serovars a, b, and c contained 4 to 6, 10 to 12, and 4 to 6 faintly stained Schiff-positive bands, respectively. The glycopolypeptides of the OMs of the three serovars were found to have relative molecular masses of 15 to 66 kDa (Fig. 4). It is recognized that in the SDS-PAGE system, glycoprotein electrophoretic mobilities may not reflect reliable molecular masses (41). A known glycoprotein, horseradish peroxidase, was used as a positive control for the periodic acid Schiff reagent reaction. Omission of either the periodic acid treatment or Schiff reagent yielded negative results. The A moieties of the OMs of the three serovars showed common bands at approximately the 31-, 38-, and 55-kDa-molecular-mass scale. The NA moieties of the OMs of serovars a, b, and c had common bands at the 43- and 70-kDa scale.

These experiments also indicated that there is variation in terms of number, location, or concentration of glycopolypeptides in the A and NA moieties of each serovar as well as between the three serovars of *T. denticola*. For example, there appears to be a higher level of a 31-kDa glycopolypeptide in the A moiety of serovar b than in the A moieties of serovars a and c. Similarly, the 26-, 34-, 48-, and 60-kDa glycopolypeptides were found only in the A moiety of serovar b. The A moiety of serovar c also lacked the 66-kDa glycopeptide.

**Apolipopolypeptide staining.** To determine the presence of

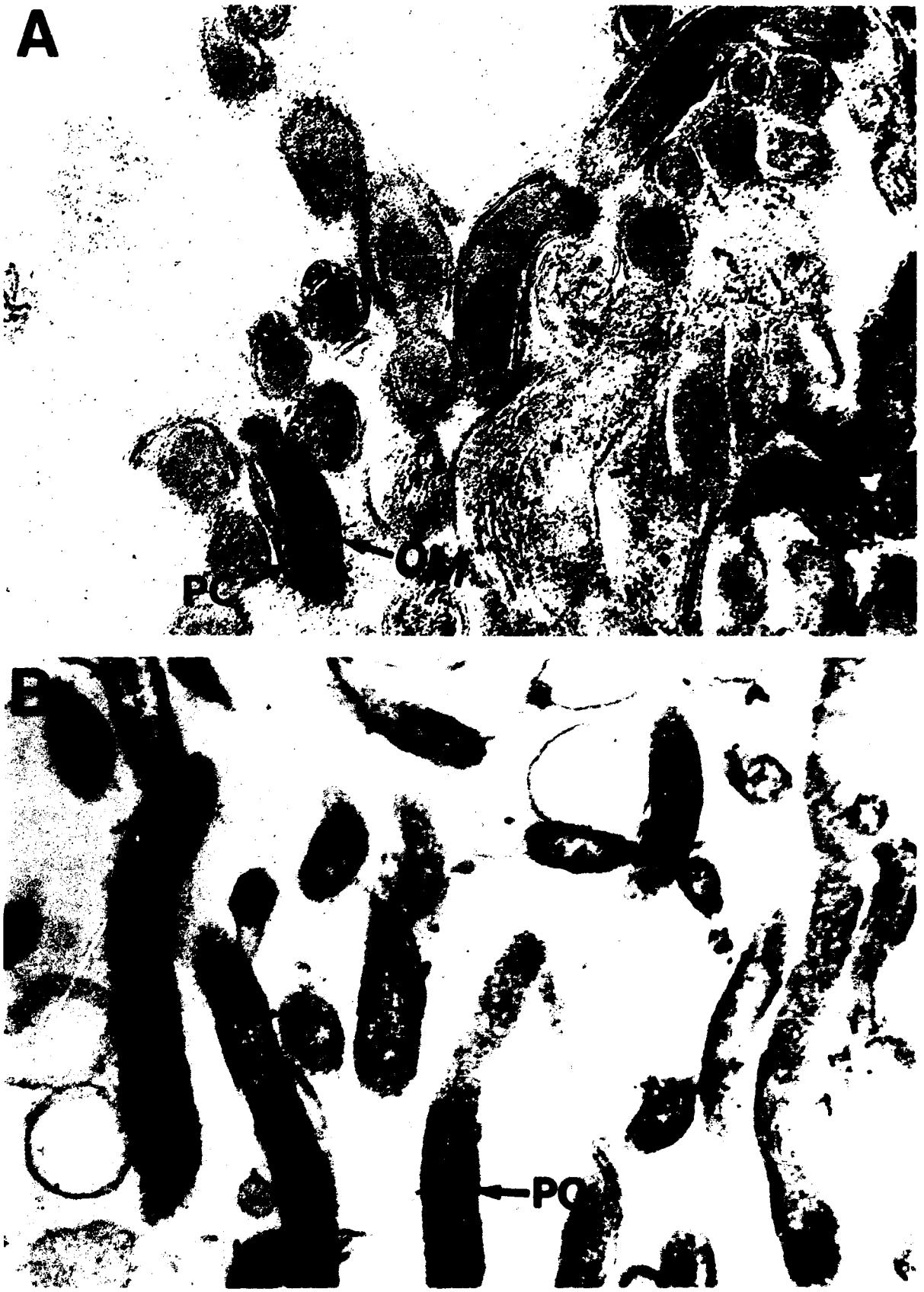


FIG. 1. (A) Normal *T. denticola* cells a, b, or c (section) serovar. (B) *T. denticola* after treatment with 1.4 mM SDS. Note the absence of the OM on the protoplasmic cylinder. (C) The A moiety of the OM obtained by dialysis against 20 mM $MgCl_2$. (D) The NA moiety of the OM of *T. denticola*. OM, outer membrane; PC, protoplasmic cylinder. Magnifications, ×67,200.

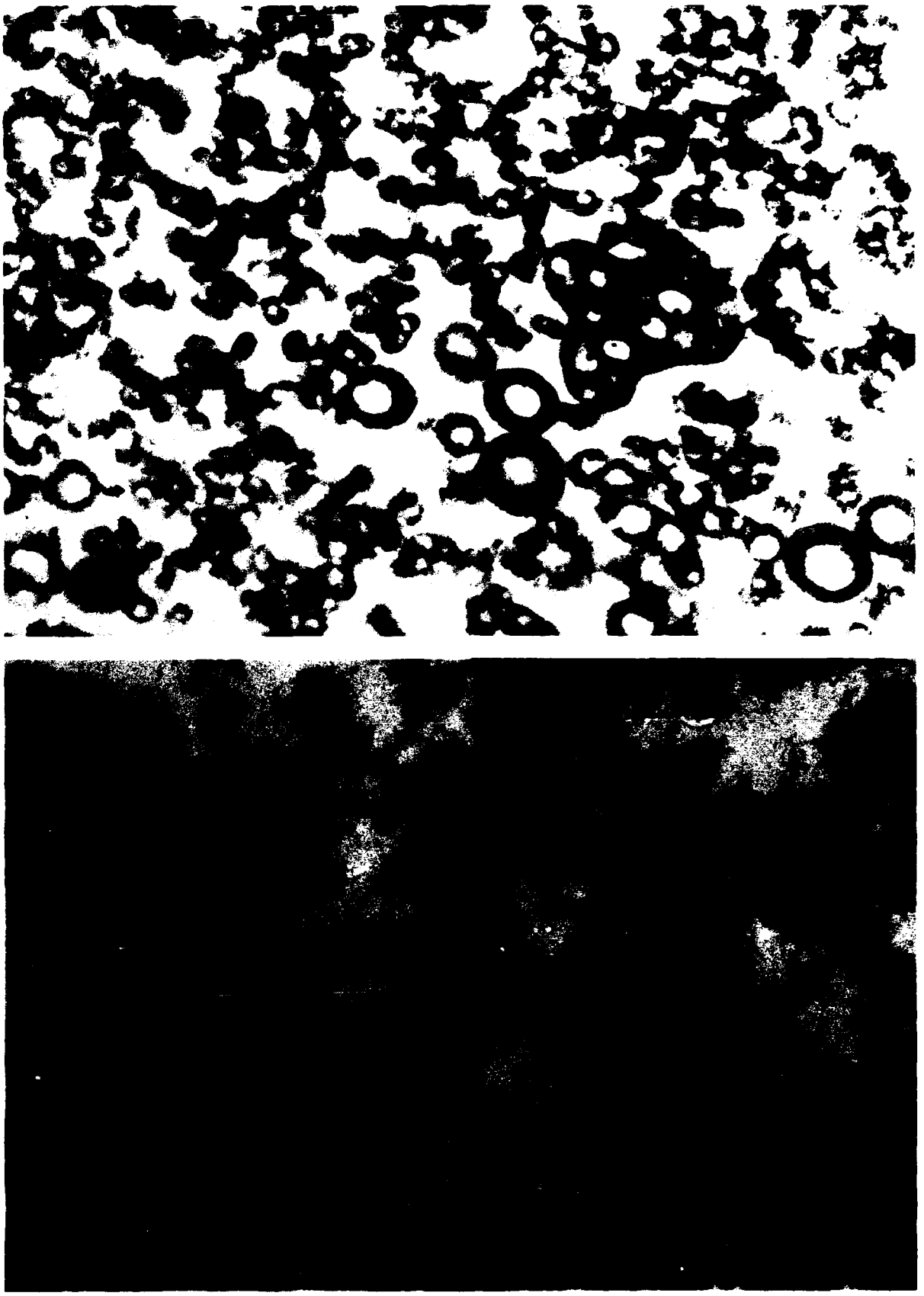

FIG. 1—*Continued.*

TABLE 1. Homogeneity of the OMs of *T. denticola* serovars a, b, and c

| Possible source of contamination | Marker[a] | Sample size[b] (μg of protein) | Sensitivity of assay |
|---|---|---|---|
| Cell wall | Muramic acid | 300 | 5 μg |
| Cytoplasmic membrane | ATPase | 300 | 0.15 μg of released P |
| Cytoplasmic contents | DNA | 200 | 2.5 μg (diphenyl reaction) |
| | DNA | 100 | 10 ng (electrophoresis) |
| | RNA | 300 | 10 μg (orcinol reaction) |
| | Hexokinase | 300 | 1 μM |

[a] None of the markers was detected.
[b] Equal quantities of the A and NA moieties of the OMs were tested in five consecutive OM preparations.

apolipopolypeptides in the OM of *T. denticola*, the resolved polypeptide components were stained with Sudan black following SDS-PAGE. Other investigators (10), using Sudan black for the identification of serum lipoproteins, have demonstrated the usefulness of this stain.

The NA moieties of the OMs of serovars *a*, *b* and *c*, upon SDS-PAGE and staining of the gels, yielded two, seven, and two Sudan black-positive bands, respectively. The bands appeared faint. The A moieties of the OMs of *T. denticola* contained four, five, and three Sudan black-positive bands for serovars a, b, and c, respectively. Lipase treatment of the OM moieties prior to SDS-PAGE reduced greatly or eliminated the Sudan black-positive bands. With both the A and NA moieties of the OMs of the three serovars, the Sudan black-positive bands appeared within the 15- to 66-kDa relative-molecular-mass range. The NA moiety of serovar b formed faint Sudan black-positive bands at the 16-, 29-, 36-, 42-, and 48-kDa relative-molecular-mass scale, while the same moiety of serovar a formed only faint Sudan-positive bands at the 36- and 42-kDa area. The A moieties of the OMs of the three serovars had common Sudan black-stained bands at the 29-, 36-, and 48-kDa scale. Serovars a and b shared a 66-kDa band. However, serovar b had an additional 42-kDa band.

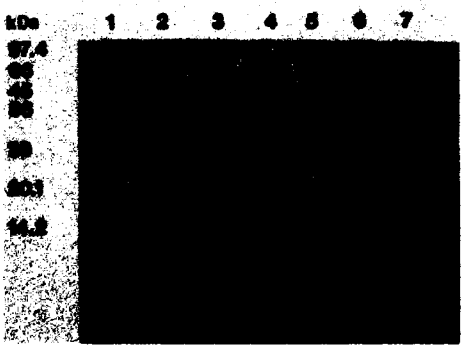


FIG. 2. SDS-PAGE analysis of the OM polypeptides of *T. denticola* serovars a, b, and c stained with Coomassie blue. Lane 1, molecular mass markers; lanes 2 through 4, the NA moieties of the OMs of serovars a, b, and c, respectively; lanes 5 through 7, the A moieties of the OMs of serovars a, b, and c, respectively. Arrows indicate the polypeptides described in the text.

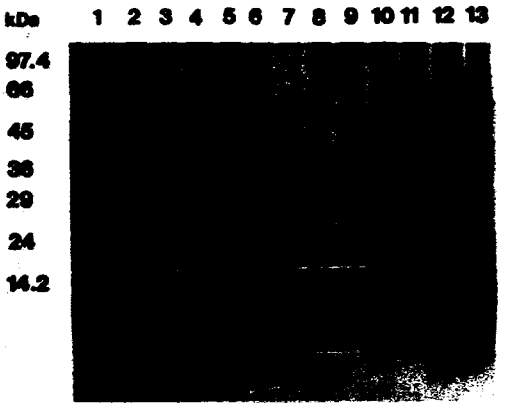


FIG. 3. SDS-PAGE analysis of the outer membrane of *T. denticola* serovars a, b, and c stained with silver nitrate. Lane 1, molecular mass markers; lanes 2 and 3, 4 and 5, and 6 and 7, charged with samples of 15 or 7.5 μg of protein of the NA moieties of the OMs of serovars a, b, and c, respectively; lanes 8 and 9, 10 and 11, and 12 and 13, charged with samples of 15 or 7.5 μg of protein of the A moieties of the OMs of serovars a, b, and c, respectively. Arrows indicate the polypeptides described in the text.

**LPS staining.** Pronase or proteinase K digestion of the NA moieties of serovars a, b, and c yielded about two, six, and five LPS bands, respectively, with relative molecular masses of 15 to 66 kDa. The 66- and 53-kDa bands were present in serovars b and c but were absent in serovar a, while a band with an approximate relative molecular mass of 45 kDa was present in serovar c but not in serovars a or b. The 66- and 53-kDa bands of serovar b were more prominent than those for serovar c were. The 66- and 53-kDa LPS bands were found in the three serovars of the A moieties, but were so

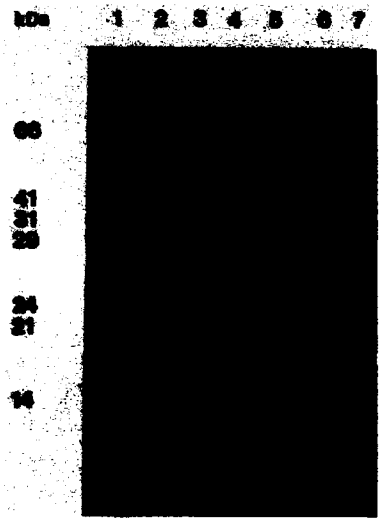


FIG. 4. Periodic acid-Schiff staining of the OM polypeptides of *T. denticola* serovars a, b, and c following SDS-PAGE. Each lane was charged with a 150-μg protein sample. Lane 1, a known glycoprotein control, peroxidase; lanes 2 through 4, charged with the NA moieties of the OMs of serovars a, c, and b, respectively; lanes 5 through 7, charged with the A moieties of the OMs of serovars a, c, and b, respectively. Arrows indicate the polypeptides described in the text.

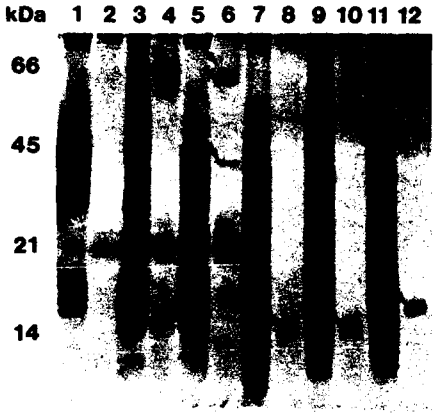


FIG. 5. LPS silver staining after pronase digestion of the outer sheath of *T. denticola* serovars a, b, and c. Lanes 1 and 2, 3 and 4, and 5 and 6, charged with samples containing 15 μg of protein of the pronase-untreated and -treated NA moieties of the OMs of serovars a, b, and c, respectively; lanes 7 and 8, 9 and 10, and 11 and 12, charged with samples containing 15 μg of protein of the pronase-untreated and -treated A moieties of serovars a, b, and c, respectively. Arrows indicate the polypeptides described in the text.

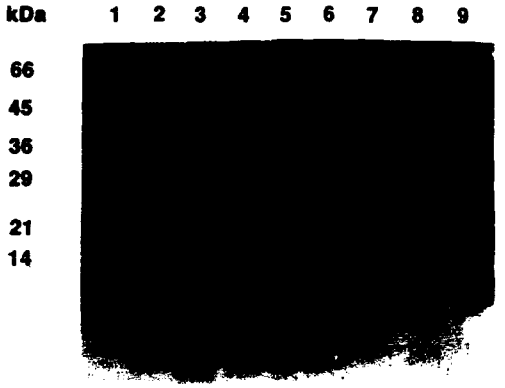


FIG. 6. LPS silver staining after hot phenol extraction of the OMs of *T. denticola* serovars a, b, and c. Lane 1, molecular mass standards; lanes 2 through 4, LPS extracted from samples containing 200 μg of protein of the NA moieties of the OMs of serovars a, b, and c, respectively; lanes 5 through 7, LPS extracted from samples containing 200 μg of protein of the A moieties of the OMs of serovars a, b, and c, respectively; lane 8, charged with 0.4 μg of *E. coli* O55:B5 LPS that had been extracted with hot phenol; lane 9, charged with 0.4 μg of *E. coli* O55:B5 LPS. Arrows indicate the polypeptides described in the text.

faint that they could not be photographed. Several bands with relative molecular masses of 10 to 15 kDa were very prominent in the A moieties of the three serovars, and they appeared very faint or absent in the NA portion of the outer membranes of serovars a, b, and c (Fig. 5).

To improve the resolution of the LPS bands described above, hot phenol extraction of the LPS and SDS-PAGE experiments were conducted. The NA moiety of serovar b had a distinct band with a relative molecular mass of 24 kDa that was absent from serovars a and c. The 66- and 53-kDa bands were found in both the NA and A moieties of serovars a, b, and c as well as the *E. coli* LPS, which was used as a control. It should be pointed out that the hot phenol LPS extraction procedure eliminated the 10-kDa band found in LPS preparations involving the use of proteolytic enzymes (Fig. 6).

**Two-dimensional protein electrophoretic studies.** To obtain a more precise account of the number, the isoelectric points, and the relative molecular masses of the OM polypeptides, two-dimensional polypeptide analysis experiments were conducted. These experiments showed an excess of 100 silver-stained polypeptides with isoelectric points of 4.2 to 6.9 and relative molecular masses of 14 to 97.4 kDa (Fig. 7). Similar relative molecular masses and isoelectric point profiles of the OM polypeptides were observed for serovars a and c.

***Limulus* assays.** Gelation of the *Limulus* amebocyte lysate has been used as a sensitive means for the detection of endotoxin (25). Since the electrophoretic studies indicated that LPS was present in the A and NA moieties of the OMs of *T. denticola*, *Limulus* tests were conducted to obtain additional evidence for the presence of endotoxin in the OM of *T. denticola*. Table 2 summarizes the results of these experiments. It is apparent that both the A and NA moieties of the OMs of serovars a, b, and c showed *Limulus* amebocyte lysate clotting activity. The A moieties had gelation endpoints in the 5-ng range, while the NA moieties, with the exception of serovar b, had gelation endpoints of 5 to 15 ng. The *E. coli* endotoxin control had a gelation endpoint of 0.5 to 1 ng.

**Chick embryo lethality assays.** Chick embryos have been used to assay the biological properties of endotoxins (36). We conducted chick embryo assays to extend our work on the LPS found in the OMs of *T. denticola*. The data obtained from these assays are given in Table 3. With the exception of serovar c, doses of 48 to 80 μg of the NA moieties and 200 to over 400 μg of the A moieties of the OMs of *T. denticola* were required to kill the 11-day-old chick embryos. *E. coli* LPS had a lethal dose close to that found for the A moieties of serovars a and b.

## DISCUSSION

The macromolecules of the spirochetal cell surface may be used for the identification of the spirochetes. Furthermore, they may be toxic to gingival cells, or they may be involved in the attachment of spirochetes to the oral tissue. The biochemical properties of the OMs of *T. denticola* serovars a, b, and c and their moieties remain unknown. Mild treatment of *T. denticola* with 1.4 mM SDS produced OMs with undetectable contamination from the cytoplasm, cell membrane, cell wall, or other recognizable spirochetal components, as judged by chemical, enzymatic, and electron microscopic examinations (Table 1, Fig. 1).

Exposure of oral spirochetes to 1.4 mM SDS solubilized the OM. However, aggregation of the OM by 20 mM $MgCl_2$ depends upon the microorganism under study (20). Thus, to avoid errors in the polypeptide profile of the OM, electrophoretic experiments with both the A and the NA moieties of the OM were conducted.

The data presented in this report indicate that the OM of the assay oral spirochetes contain simple proteins as well as glycoproteins and lipoproteins. Although the OM polypeptide profiles of the three serovars of *T. denticola* were similar in many respects, there appeared to be a certain diversity with regard to the concentration, number, or location of the various polypeptides within the electropherograms. Isolation of these unique polypeptides may provide us with some macromolecules that could be used in studies concerned

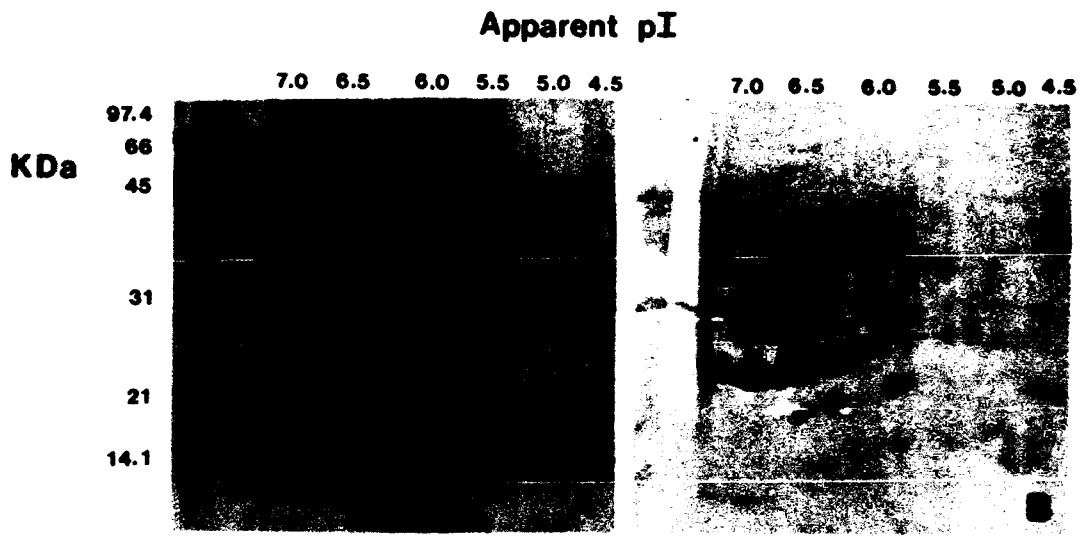


FIG. 7. Silver-stained two dimensional polypeptide profile of the A (A) and the NA (B) moieties of the OM of *T. denticola* serovar b. Sample concentration, 250 μg of protein.

with the biological or diagnostic functions of the OM polypeptides.

SDS-PAGE followed by Coomassie brilliant blue R-250 staining of whole-cell lysates of *T. denticola* serovar c by Umemoto et al. (40) revealed a 53-kDa antigen that was absent in whole-cell lysates of *T. denticola* serovars a and c. The polypeptide bands of the whole-cell lysates ranged from approximately 15 to 140 kDa. *T. denticola* serovars a and c had similar Coomassie brilliant blue R-250-stained SDS-PAGE profiles, with the main bands being approximately 50 kDa. Immunoelectron microscopic studies suggested that the 53-kDa antigen of *T. denticola* serovar c may originate from the OM. The use of monoclonal antibodies and immunoelectron microscopy has also resulted in the demonstration of a surface-specific antigen for *T. denticola* serovar "b" by Simonson et al. (34). This antigen appeared to be specific for *T. denticola* serovar b because the monoclonal antibody raised by Simonson et al. (34) did not cross-react with *T. denticola* serovar c by enzyme-linked immunosorbent assay (40).

A common observation that was made during the electrophoretic studies of the OM of *T. denticola* was the tendency of *T. denticola* serovar b to have more polypeptides (Fig. 1 to 6) or electrophoretic polypeptide profiles slightly different from those of *T. denticola* serovars a or c.

The finding that the OM of *T. denticola* serovars a, b, and c contained periodic acid Schiff-positive reagent bands (Fig. 4) suggests that glycopeptides may be present in the OMs of *T. denticola* serovars a, b, and c and that they may be involved in the attachment of the assay spirochetes to human gingival cells. *T. denticola* has been shown to adhere to various human cells (32, 42). Weinberg and Holt (42) have shown that *T. denticola* GM-1 and MS25 were more adherent to human gingival fibroblasts than *T. denticola* TD-4 (serovar c) was. Polyclonal antibodies to strain GM-1 inhibited GM-1 adherence by 70%, while strains MS25 and TD-4 showed different degrees of cross-reactive inhibition, indicating common but not identical epitopes on the surfaces of the three *T. denticola* strains. Pretreatment of the three strains with trypsin did not inhibit adherence; however, exposure to sugars and lectin pretreatment of the human

TABLE 2. *Limulus* amebocyte lysate clotting activity of the outer membrane of *T. denticola*[a]

| Sample concn (ng) | *Limulus* lysate clotting activity[b] | | | | | |
|---|---|---|---|---|---|---|
| | Serovar a | | Serovar b | | Serovar c | |
| | A moiety | NA moiety | A moiety | NA moiety | A moiety | NA moiety |
| 225 | + | + | + | + | + | + |
| 113 | + | + | + | + | + | + |
| 52 | + | + | + | + | + | + |
| 26 | + | + | + | + | + | + |
| 11 | + | − | + | + | + | − |
| 5.5 | + | − | + | + | + | − |
| 2.8 | − | − | − | + | − | − |
| 1.4 | − | − | − | − | − | − |
| 0.7 | − | − | − | − | − | − |
| 0 | − | − | − | − | − | − |

[a] Data were obtained from seven determinations.
[b] +, solid clot in the bottom of the tube; −, no clot.

TABLE 3. Chick embryo lethality of the outer membrane of *T. denticola*

| Material inoculated[a] | No. inoculated | Concn (μg) yielding 100% mortality |
|---|---|---|
| A moiety, serovar a | 27 | 210–315 |
| NA moiety, serovar a | 22 | 68–80 |
| A moiety, serovar b | 28 | 316–407 |
| NA moiety, serovar b | 25 | 48–60 |
| A moiety, serovar c | 23 | >400 |
| NA moiety, serovar c | 21 | 300–409 |
| *E. coli* LPS | 19 | 50–100 |
| Control (no OM) | 17 | No deaths |

[a] The indicated material was diluted in pyrogen-free water and was inoculated on the chorioallantoic membrane of 11-day-old chick embryos.

gingival cells inhibited adherence. These results implicate a lectin-like adhesion on *T. denticola* surface with activity for sugars on the human gingival fibroblasts (42).

Both the A and NA moieties of the OM of *T. denticola* serovar b contained more apolipopeptides than *T. denticola* serovar a or c did, as judged by Sudan black staining. The differences in the number of apolipopeptides observed could not be explained on the basis of differences in the staining properties of the apolipopeptides of the various serovars, because apolipoprotein staining with Sudan black appeared to be generally modest. Therefore, some of the diversity in the apolipopeptide profiles observed is probably serovar specific.

The presence and role of LPS in spirochetes remains conjectural (9). There is evidence for the presence of LPS in the *Treponema refringens* Nichols strain (18). The presence, location, or function of LPS in *T. denticola* is not known.

The detection of LPS-like components (Fig. 5 and 6, Tables 2 and 3) in the OM of *T. denticola* is another interesting finding brought out by this study. An LPS that is also known as endotoxin has been used for diagnostic purposes, and it may elicit a wide variety of pathophysiological effects during an infection (38). However, further investigations will be required to define the precise role of the LPS-like component of the OM of *T. denticola*.

## ACKNOWLEDGMENTS

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Short communication

# Effect of outer membrane of *Treponema denticola* on bone resorption

**C. Gopalsami[1], W. Yotis[1], K. Corrigan[2], S. Schade[1], J. Keene[3], L. Simonson[4]**

Departments of: [1]Microbiology and Immunology, [2]Radiation Control and [3]Periodontics, Loyola University of Chicago, Maywood, [4]Naval Dental Research Institute, Great Lakes, Illinois, USA

*Gopalsami C, Yotis W, Corrigan K, Schade S, Keene J, Simonson L. Effect of outer membrane of* Treponema denticola *on bone resorption. Oral Microbiol Immunol 1993: 8: 000–000.* 

The effect of the outer membrane (outer sheath) of *Treponema denticola* on bone resorption was studied. Bone resorption was measured by the release of previously incorporated $^{45}Ca$ from the shafts of the radii and ulnae of 19-day fetal rats. A treated-over-control ratio (T/C ratio) significantly greater than 1 indicated the stimulation of bone resorption by the test substance. The addition of outer membrane of *T. denticola* increased the release of $^{45}Ca$ from the assay bones. The minimum concentrations required to yield significant $^{45}Ca$ release from the assay bones were 15, 22 and 75 μg protein/ml for serovars a, b and c, respectively. These protein values corresponded to estimated lipopolysaccharide (LPS) contents of 0.6, 0.8 and 2.8 μg/ml, based on 3-deoxy-2-*manno*-octulosonate (KDO) analysis. Heat treatment of outer membrane (60° for 30 min) did not change the effect on $^{45}Ca$ release. Parathyroid hormone or prostaglandin $E_2$, known to act synergistically with lipopolysaccharides in bone resorption, was also added to the assay system. Neither prostaglandin $E_2$ at $10^{-7}$ M nor parathyroid hormone at 40 ng/ml, by itself, increased $^{45}Ca$ release. However, in the presence of 10 μg protein/ml of outer membrane of serovar b at 120 h, the T/C ratio was increased to $1.31 \pm 0.07$ and $1.58 \pm 0.118$, respectively. These results suggest that an LPS-like material is present in the outer membrane of *T. denticola* that may be responsible for bone resorption in the *in vitro* system.

Key words: *Treponema denticola*; outer membrane; $^{45}Ca$ release; bone resorption; embryonic rat bone; lipopolysaccharide; oral spirochete

William W. Yotis, Ph.D., Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University of Chicago, 2160 S. First Avenue, Maywood, IL 60153, USA

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Bone resorption is one of the major features of advanced periodontal diseases (3, 4). The microflora associated with bone loss is heterogeneous and highly complex (12, 14, 15, 19), but among the bacteria that are invariably present are spirochetes. These are located more often at diseased sites than healthy sites and are in greater concentrations in subgingival plaque (1, 11, 21). *Treponema denticola* is one of the best characterized oral spirochetes, and Simonson et al. (21) quantified *T. denticola* in human clinial studies using monoclonal antibodies.

A variety of factors appear to contribute to the tissue destruction in chronic periodontal disease (5, 9). Among the microbial factors studied, bacterial endotoxins (lipopolysaccharides), which are common to all gram-negative bacteria, exhibit significant bone resorption activity (6). Previous studies in our laboratory indicated the presence of lipopolysaccharides in the outer membrane of *T. denticola* (23). In addition, our results using 3-deoxy-2-*manno*-octulosonate (KDO) analysis indicated that lipopolysaccharide may be present at the level of 0.2–0.5% by weight in *T. denticola* whole bacteria (20). These findings prompted us to extract the outer membrane of *T. denticola* and to study its potential to stimulate bone resorption. We report in this article that the outer membrane has a potent stimulatory effect on bone resorption; we determined this using an assay in which $^{45}Ca$ is released from fetal rat bones.

*T. denticola*, ATCC strains 35405, 33521 and 35404 (serovars a, b and c, respectively) were cultured anaerobically at 35°C for 5–8 days in the pre-reduced GM-1 medium prepared according to Blakemore & Canale-Parola (2); the medium was supplemented with 5% (vol/vol) heat-inactivated rabbit serum. Cells from 15 liters of the assay treponemes in the late logarithmic phase of growth were removed by centrifugation at 12,000 × g for 30 min, washed twice with Milli Q water (endotoxin-free) and lyophilized.

Timed pregnant CD rats were obtained from Sprague Dawley Co., Indianapolis, IN. $BGJ_b$ medium and prostaglandin $E_2$ ($PGE_2$) were purchased from Sigma Chemical Co., St. Louis, MO. Parathyroid hormone amino acid residues 1–34 (bovine) was procured from Calbiochem, La Jolla, CA. Radioactive calcium [$^{45}CaCl_2$] was purchased from Amersham Corp., Arlington Heights, IL (specific activity 10–40 mCi/mg calcium). Pefabloc® SC, a protease inhibitor, was obtained from Pentapharm, Basel, Switzerland.

The outer membranes of *T. denticola* were prepared following a slight modification of protocols of Johnson et al. (10) and Wachter & Johnson (22). Lyophilized cells (250 mg) of late log phase of *T. denticola* were extracted with 100 ml of 0.7 mM sodium dodecy (sulfate) (SDS) at room temperature for 15 min in the presence of the protease inhibitor 0.1 mM Pefabloc. The cells were centri-

fuged at 12,000 × g for 20 min and the supernatant was filtered through a 0.2-μm cellulose nitrate membrane filter. The filtrate was dialyzed in Spectra Por cellulose ester membrane dialysis tubing of 2000 Mr cutoff against 20 mM $MgCl_2$ at 4°C for 3 days with 3 changes of dialysis fluid. After dialysis, the filtrate was concentrated with polyethylene glycol to 5 ml and frozen in aliquots until used. The absence of muramic acid, adenosine triphosphatase, hexokinase and nucleic acid as well as electron microscopy indicated that the outer membrane preparations were homogeneous (23). Protein estimation followed the protocol of Hill & Straka (8) and SDS estimation that of Pitt-Rivers & Impiombato (16).

The bone resorption assay system developed by Raisz et al. (18) was essentially followed. Pregnant rats were injected subcutaneously with 200 μCi $^{45}CaCl_2$ in 0.3 ml of saline on the 18th day of gestation. One day later, the rats were killed by $CO_2$ anaesthesia. The fetal radii and ulnae were removed by microdissection and explanted as pairs in 24-well cell culture dishes (left radius and ulna versus right radius and ulna) for organ culture in 0.5 ml of $BGJ_b$ medium, supplemented with 5% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 0.5 μg/ml fungizone. Cultures consisting of 2 bones were incubated for 24 h at 37°C in 6% $CO_2$ and air, without the test substance, so that much of the surface $^{45}Ca$ could exchange with the medium. After 24 h, one set of bones (ulna plus radius) was transferred to medium alone and the paired set was transferred to medium containing *T. denticola* outer membrane. All cultures were incubated for 48 h and 120 h with a change of medium after 48 h. At the specified time intervals, 0.2-ml samples of culture media were pipetted off each well; 10 ml of ready protein liquid scintillation cocktail (Beckman Instruments, Fullerton, CA) was added and the radioactivity counted in Beckman liquid scintillation counter LS 5802. Bone resorption was assessed by the release of incorporated $^{45}Ca$ from the shafts of the radii and ulnae. The ratio of the release of $^{45}Ca$ from bones treated (T) with outer membrane to the release of $^{45}Ca$ from paired control (C) bones was used to measure bone resorption. A mean T/C ratio significantly greater than one indicated stimulation of bone resorption by the test substance. Statistical significance was assessed by the paired Student's *t*-test.

To study the effect of concentration of outer membrane on $^{45}Ca$ release, various protein concentrations of whole outer membrane preparations of *T. denticola* were added to $^{45}Ca$ prelabeled embryonic bones in culture and the $^{45}Ca$ release was studied. Table 1 gives the data on $^{45}Ca$ release; the addition of outer membrane increased $^{45}Ca$ release at 48 h. In the case of *T. denticola* serovars a and b, significant $^{45}Ca$ release was observed when the concentration of outer membrane was 15 and 22 μg protein/ml, respectively, yielding T/C ratios of 1.61 ± 0.19 and 1.47 ± 0.10, respectively; but at concentrations of 75–108 μg protein/ml, the $^{45}Ca$ release dropped to that of control, indicating possible damage to the tissue cells. For *T. denticola* serovar c, the minimum concentration to produce significant $^{45}Ca$ release was 75 μg protein/ml. These protein values corresponded to estimated LPS contents of 0.6, 0.8 and 2.8 μg/ml, based on KDO analysis (20). With very low concentrations (< 10 μg protein/ml) significant $^{45}Ca$ release was not obtained.

When sham extracts were added, the

*Table 1.* Effect of the outer membrane of *T. denticola* on $^{45}Ca$ release from embryonic bones in culture

| Outer membrane | Protein (μg/ml) | Estimated lipopolysaccharide[b] (μg/ml) | $^{45}Ca$ release cpm/0.2 ml | | $^{45}Ca$ release T/C ratio |
|---|---|---|---|---|---|
| | | | Control (C) | Treated (T) | |
| ATCC 35405 | 3.75 | 0.15 | 4971 ± 1035[a] | 5615 ± 281 | 1.20 ± 0.16 |
| (serovar a) | 7.5 | 0.3 | 3246 ± 457 | 3943 ± 905 | 1.19 ± 0.13 |
| | 15.0 | 0.6 | 2766 ± 468 | 4248 ± 523 | 1.61 ± 0.19** |
| | 30.0 | 1.2 | 3650 ± 348 | 5264 ± 559 | 1.45 ± 0.10* |
| | 75.0 | 3.0 | 3656 ± 379 | 4035 ± 157 | 1.16 ± 0.09 |
| ATCC 33521 | 5.4 | 0.2 | 3786 ± 546 | 3797 ± 760 | 1.04 ± 0.13 |
| (serovar b) | 10.8 | 0.4 | 3920 ± 246 | 4153 ± 264 | 1.07 ± 0.05 |
| | 21.6 | 0.8 | 2710 ± 249 | 3937 ± 457 | 1.47 ± 0.10*** |
| | 43.2 | 1.6 | 3283 ± 302 | 4439 ± 333 | 1.41 ± 0.12*** |
| | 108.0 | 4.0 | 4077 ± 899 | 3765 ± 476 | 1.09 ± 0.28 |
| ATCC 35404 | 3.75 | 0.14 | 5723 ± 495 | 4896 ± 440 | 0.86 ± 0.02 |
| (serovar c) | 7.5 | 0.28 | 5253 ± 516 | 5680 ± 958 | 1.07 ± 0.07 |
| | 15.0 | 0.56 | 4390 ± 717 | 4389 ± 667 | 1.02 ± 0.09 |
| | 30.0 | 1.12 | 3691 ± 175 | 4034 ± 205 | 1.11 ± 0.09 |
| | 75.0 | 2.8 | 3928 ± 247 | 4933 ± 470 | 1.28 ± 0.12* |
| Outer membrane | 5.4 | 0.2 | 5050 ± 573 | 5226 ± 833 | 1.03 ± 0.09 |
| serovar b | 10.8 | 0.4 | 4249 ± 206 | 4187 ± 215 | 0.99 ± 0.07 |
| 60° for 30 min. | 43.2 | 1.6 | 2854 ± 300 | 3814 ± 335 | 1.34 ± 0.04** |
| *E. coli* | | 1 | 4109 ± 1533 | 4505 ± 1051 | 1.24 ± 0.20 |
| 055:B5 LPS | | 5 | 4506 ± 338 | 6286 ± 341 | 1.41 ± 0.09*** |
| Sham extract | – | | 3672 ± 315 | 3333 ± 270 | 0.91 ± 0.03 |
| Bones heated at 75° for 4 min | | | 2326 ± 269 | 2003 ± 63 | 0.87 ± 0.04 |

Mean ± SEM [a]Based on KDO analysis, assuming that *T. denticola* lipopolysaccharide contains 2.3% KDO, as does *E. coli* 055:B5 lipopolysaccharide. Each value is the mean of 6 to 8 pairs of cultured bones.

*$P < 0.05$, **$P < 0.02$ and ***$P < 0.01$, based on a control T/C ratio of 1.00.

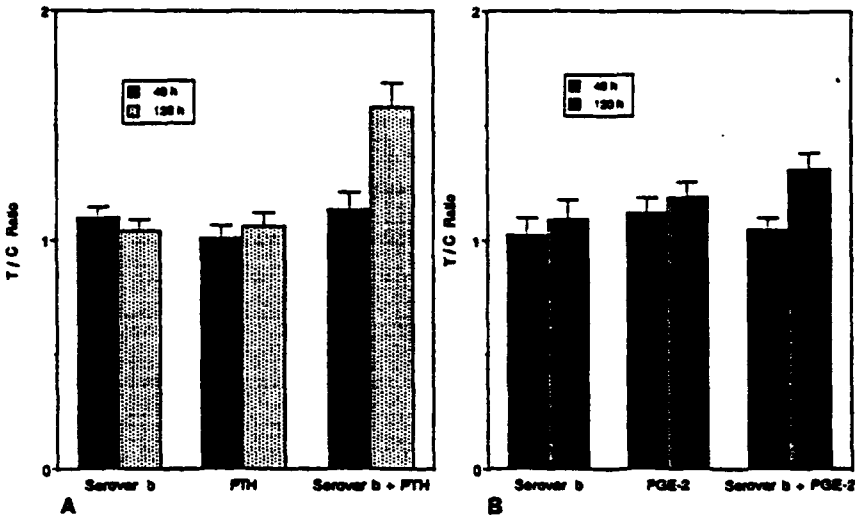


*Fig. 1.* A. Effect of *T. denticola* serovar b outer membrane (10 μg protein/ml) in the presence of a suboptimal concentration of parathyroid hormone (PTH, 40 ng/ml) on $^{45}Ca$ release from rat embryonic bones. Treated/control (T/C) ratios and their SEM are plotted, for outer membrane from serovar b alone (serovar b), for PTH alone (PTH) and for outer membrane and PTH together (serovar b+PTH). B. Effect of *T. denticola* serovar b outer membrane (10 μg protein/ml) in the presence of a suboptimal concentration of prostaglandin $E_2$ (PGE-2, $10^{-7}$ M) on $^{45}Ca$ release from rat embryonic bones. Treated/control (T/C) ratios and their SEM are plotted, for outer membrane from serovar b alone (serovar b), for PGE-2 alone (PGE-2) and for outer membrane and PGE-2 together (serovar b+PGE-2).

T/C ratios remained at $0.91 \pm 0.03$. When bones were heated at 75°C for 4 min and used as a negative control, the T/C ratios were found to be $0.87 \pm 0.04$. As a positive control, the addition of 5 μg/ml of purified LPS of *Escherichia coli* yielded a T/C ratio of $1.41 \pm 0.09$. Heating the outer membrane preparation at 60° for 30 min prior to addition to bone cultures did not change the effect on $^{45}Ca$ release (Table 1). This finding suggests that the bone resorption-stimulating factor present in the outer membrane of *T. denticola* was heat stable, as would be expected of lipopolysaccharides. It is worthy of note that *T. denticola* serovar b contained about 2 times more KDO, a specific marker for lipopolysaccharides, in the outer membrane preparation than serovars a and c. Therefore, for subsequent studies, ATCC strain 33521 (serovar b) was used. To rule out the possible inhibitory effect of residual SDS that might be present in these outer membrane preparations, the quantity of SDS was determined. The values ranged from 0.009 to 0.030 μg SDS per mg protein and the mean ratio was $0.018 \pm 0.007$ (w/w). The maximum quantity used was 108 μg protein. Under these conditions it was found that SDS levels of 1.8 μg or 0.0004% was detected. These SDS concentrations in the outer membrane were far below the inhibitory level of SDS on bone resorption, as reported by Millar et al. (13).

To strengthen the hypothesis that the active component in the outer membrane of *T. denticola* is a lipopolysaccharide, the effect of parathyroid hormone and prostaglandin $E_2$, both of which are known to act synergistically with lipopolysaccharides, was tested. The suboptimal concentrations of *T. denticola* outer membrane (10 μg protein/ml), PTH [40 ng/ml] (17) and $PGE_2$ [$10^{-7}$ M] (18) were chosen and added individually to various sets of bones in culture. Six to eight replicate cultures, each with paired controls, were assayed for all concentrations tested. Release of $^{45}Ca$ was measured at 48 h and 120 h and the results are presented in Fig. 1. The outer membrane of *T. denticola* serovar b at a concentration of 10 μg protein per ml and lower yielded the same $^{45}Ca$ release from the rat bones as the controls. That is, the T/C ratios were $1.100 \pm 0.049$ and $1.040 \pm 0.051$ at 48 h and 120 h, respectively. Parathyroid hormone at a concentration of 40 ng per ml also produced the same result in the bone resorption assay system. However, when the outer membrane of *T. denticola* serovar b and parathyroid hormone were added together to the bone resorption assay system at these levels, $^{45}Ca$ release from the bones was enhanced significantly at 120 h, yielding a T/C ratio of $1.580 \pm 0.108$, but not at 48 h of incubation (Fig. 1A). Addition of $PGE_2$ at very low levels ($10^{-7}$ M) did not stimulate $^{45}Ca$-prelabeled fetal rat bones. However, when bones were exposed to outer membrane of serovar b at 10 μg protein/ml together with $PGE_2$ at $10^{-7}$ M, significant $^{45}Ca$ was released at 120 h (T/C ratio = $1.311 \pm 0.073$) (Fig. 1B). These results are in agreement with the data reported by Hausmann et al. (7) and Raisz et al. (18), who showed synergistic action between the bacterial endotoxins and hormones on $^{45}Ca$ release at 120 h of incubation.

The results presented here show that outer membranes extracted from *T. denticola* by 0.7 mM SDS can stimulate the release of previously incorporated $^{45}Ca$ from embryonic rat bones in tissue culture. This is the first report describing bone-resorbing activity of *T. denticola*. A recent review by Hopps & Sisney-Durrant (9) gave information on different bacterial factors (lipopolysaccharides, lipoteichoic acid, bacterial lipoproteins, fatty acids and peptidoglycan, a capsular material from *Actinobacillus actinomycetemcomitans*) that stimulate bone-resorbing activity.

From such chemical and biological assays conducted on *T. denticola* outer membrane as *Limulus* assay, chick embryo lethality test (23), complement activation, KDO analysis and heat treatment and mitogenic assay (20), we conclude that an lipopolysaccharide-like material is present in the outer membrane of *T. denticola* that may be responsible for bone resorption in the *in vitro* system described here.

**Acknowledgements**

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# A Study of the Acid Phosphatase of Treponema denticola

C. A. NORTON HUGHES[1] and W. W. YOTIS

Department of Microbiology, Loyola University Stritch School of Medicine, Maywood, IL 60153, USA
[1] Current Address: Department of Microbiology, University of Minnesota Medical School, Minneapolis, MN 55455, USA

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## Summary

This study describes some of the properties of the acid phosphatase of the potential periodontopathogen *Treponema denticola*. The highest enzyme activity was found in 87 h old cells. Two optimum pHs for enzyme activity were detected, one at pH 4.8 and another at pH 6.2. Divalent cations did not influence the acid phosphatase of *T. denticola*. The anion $F^-$ added in the form of NaF and at a level greater than 20 µg/ml $F^-$ diminished the activity of the acid phosphatase of intact cells of *T. denticola*. The addition of 10 µg/ml $F^-$ as $SnF_2$ induced a statistically significant reduction of acid phosphatase activity.

The apparent $K_m$ for the acid phosphatase was 7.3 mM with p-nitrophenyl phosphate as substrate. Fluoride appeared to be a noncompetitive inhibitor of the enzyme with an apparent $K_i$ of 0.3 mM. Acid phosphatase may be released partially in osmotic shock fluids. Also, 7-diazonium-1, 3-naphthalene disulfonate, which is incapable of penetrating the bacterial permeability barrier and is known to inactivate enzymes found in the bacterial periplasmic place, suppressed the activity of the acid phosphatase in intact cells of *T. denticola*.

## Zusammenfassung

Die höchsten Aktivitäten der sauren Phosphatase (sP) wurden in 87 h alten Zellen von *Treponema denticola* gefunden, einem potentiell periodontopathogenen Keim. Das Enzym wies zwei pH-Optima auf, bei pH 4,8 und 6,2. Zweiwertige Kationen hatten keinen Einfluß. Ab 20 µg/ml verminderten Fluoridionen (als NaF) die sP-Aktivität intakter *T. denticola*-Zellen. Zugabe von 10 µg/ml $F^-$ als $SnF_2$ induzierte eine signifikante Reduktion der sP-Aktivität. Mit p-Nitrophenylphosphat als Substrat ergab sich eine apparente Km von 7,3 mM. Fluorid erwies sich als nicht-kompetitiver Inhibitor mit einer apparenten Ki von 0,3 mM. Osmotischer Schock kann die sP z. T. freisetzen. 7-Diazonium-1,3-naphthol-disulfonat, das bakterielle Permeabilitätsbarrieren nicht durchdringen kann, zugleich aber Enzyme im periplasmatischen Raum inaktiviert, unterdrückte entsprechend die sP intakter *T. denticola*-Zellen.

## Introduction

*Treponema denticola* is a common inhabitant of the human oral cavity. *Simonson* et al. (19) have provided evidence of a positive relationship between *T. denticola* and periodontitis.

The use of fluoride in preventive dentistry is widespread. However, studies on the effect of fluoride on potential periodontopathogens are limited (16,22,23). Our studies have shown that fluoride, at a concentration of 10–40 μg/ml reduced the growth of *T. denticola* (8).

Initial enzyme profile screening studies in this laboratory revealed that the acid phosphatase of *T. denticola* was significantly reduced by sodium fluoride (23). In general, phosphatases hydrolyze a variety of phosphatase esters (1,11). Bacteria are relatively impermeable to phosphate esters that are not actively transported (13). Substrates must be dephosphorylated for subsequent metabolism. Dephosphorylation is also associated with intracellular or extracellular movements of metabolites. The primary evidence for the periplasmic location of bacterial phosphatases is the ability of intact cells of *Salmonella typhimurium* (11), or *Escherichia coli* (3,20) to hydrolyze phosphorylated compounds. Thus, the periplasmic phosphatases could act as scavenging enzymes, degrading nontransportable phosphate esters that could then be transported and utilized by the cell. Information on the acid-phosphatase of *T. denticola* and its interaction with NaF is limited (23). This article describes a quantitative study of the acid phosphatase activity of *T. denticola.*

## Materials and Methods

*Organism and culture conditions. T. denticola* ATCC 33520 was grown in GM-1 medium as previously described (8).

*Enzyme assay.* Acid phosphatase activity was measured by a method of *Bessey* et al. (2). Briefly, the reaction mixture contained: 0.2 ml whole cell material (or whole cells or cellular fractions in 0.2 M sodium acetate buffer, pH 4.8); 0.1 ml 8 mM p-nitrophenyl phosphate (pNPP); 0.1 ml deionized distilled water. The reaction mixture was incubated for 60 min at 37 °C. The reaction was stopped with the addition of 0.8 ml 0.03 N NaOH. The resulting color change was measured at 405 nm with the intensity of color formed proportional to acid phosphatase activity. The standard used in these assays was p-nitrophenol (pNP). Activity was reported as nanomoles pNP released per mg soluble protein, BSA equivalents. No metal ion requirements were detectable for the enzyme activity in intact cells of *T. denticola.*

*Analytical procedures.* Soluble protein was measured by the method of *Lowry* et al. (15). Bovine albumin (98–99% pure) was used as the standard. Spectrophotometric determinations were measured on a Gilford Response UV-VIS scanning spectrophotometer (Ciba Corning Diagnostics Corp., Gilford Systems, Oberlin, OH). Hexokinase (EC2.7.1.1) activity was measured using an NADP-linked assay (9), which measured the increase in NADPH absorbance at 340 nm. Hexokinase, derived from Baker's Yeast, type F-300, sulfate-free, was used as a standard.

*Cell fractionation.* Cells were harvested at desired phases of growth by centrifugation for 25 min at 5,000 x g, washed with cold 10 mM Tris-HCl buffer (pH 7.3) with 30 mM NaCl, and suspended in the same buffer for enzyme assays. The washing procedure did not release detectable amounts of the enzyme into the supernatant solution.

Cell extracts were prepared from washed spirochetes that had been suspended in 10 mM Tris-HCl buffer (pH 7.3) containing 4 mM dithiothreitol. The whole cell suspensions were flushed with $N_2$ (Medical Grade, AIRCO, Murray Hill, NJ) and passed twice through the

French pressure cell (American Instrument Co., Inc., Silver Spring, MD) at 16,000 lb/in$^2$. The resulting disrupted cell suspension was labeled "cell extract". The procedures outlined by *Dassa* and *Boquet* (4) and *Dvorak* et al. (7) were used with some modifications to obtain material from the different compartments of *T. denticola*. Cultures of *T. denticola* 33520 were harvested by centrifugation (5,000 x g, 25 min, 5 °C) and washed twice with 50 mM Tris-HCl buffer (pH 7.8) containing 30 mM NaCl. Cell pellets were suspended in 50 mM Tris-HCl buffer (pH 7.8) containing 30% (w/w) sucrose and 1 mM EDTA at a concentration of 1 g wet wt/40 ml at 24 ± 1 °C. The mixture was stirred for 10 min and centrifuged. The supernatant was discarded and the plasmolyzed cells were subjected to a sudden osmotic transition by rapid dispersal in cold water (40 ml/g wet weight). The mixture was stirred for 10 min and centrifuged in the cold. The shocked bacteria were pelleted and retained. The supernatant "shock fluid" was concentrated to dryness by lyophilization. The dry powder was dissolved in a volume of distilled water equivalent to one fortieth of the original volume.

The shocked bacteria were suspended in 50 mM Tris-HCl buffer (pH 7.8) containing 10% (v/v) glycerol, 0.2 mg/ml DNAse I, 0.2 mg/ml pancreatic RNAse A and 2 mM $MgCl_2$, and passed three times through the French pressure cell at 16,000 lb/in$^2$. The unbroken cells were removed by centrifugation and the supernatant was retained for further centrifugation at 300,000 x g for 2 h at 5 °C. The resulting supernatant was decanted and labeled "cytoplasmic contents". The pellet or "membrane fraction" was suspended in buffer.

*Diazotization.* The reagent 7-diazonium-1,3-naphthalene disulfonate (diazo-NDS) was prepared by the method of *Pardee* and *Watanabe* (18) as follows: a 55 mg amount of 7-amino-1,3-naphthalene disulfonic acid (NDS; Aldrich Chemical Co., Inc., Milwaukee, WI) was dissolved in 3.5 ml $H_2O$ containing 0.05 ml concentrated HCl and was cooled in an ice-salt bath; then 0.25 ml of 0.5 M $NaNO_2$ solution was added at −3 °C. After 30 min, 2 ml of the diazo-NDS was added to a 7 ml suspension of washed stationary phase *T. denticola*. After incubation for 1 h at 24 ± 1 °C, the cells were washed by centrifugation. Shocked cells were treated in the same manner and subsequently disrupted by French pressure cell passage to obtain cell "membranes" and "cytoplasmic" fractions. Controls contained all components except diazo-NDS.

*Statistical analysis.* Results were analyzed by the Student's t-test (6). The computer software program Sigma-Plot, version 3.1 (Jandel Scientific, Sausalito, CA), was used for linear regression calculations. Whenever possible, chemicals of analytical reagent (AR) quality were used. Unless otherwise noted, all chemicals and regents were obtained from Sigma Chem. Co., (St. Louis, MO), and were of analytical grade.

## Results

### *Culture age and enzyme expression*

The physiological state of the cells may influence the synthesis of cellular enzymes. To this end, 16, 23, 39, 48, 63, 87 and 120 h cultures of *T. denticola* 33520 were assayed for acid phosphatase. Cultivation of *T. denticola* for 87–120 h yielded cells with the best acid phosphatase activity (data not shown). This time interval represents the late exponential phase of growth of *T. denticola* (8).

### *Effect of pH on acid phosphates of intact cells of T. denticola*

To determine the dependence of enzyme activity on pH, intact, early stationary-phase cells were assayed for acid phosphatase activity over a range of pH values. Two optimal pHs were observed (Fig. 1). One pH optimum between 4.6 and 4.8 was quite distinct. Another broader pH optimum centered at about pH 6.2. Only the pH 4.8 enzyme was investigated.

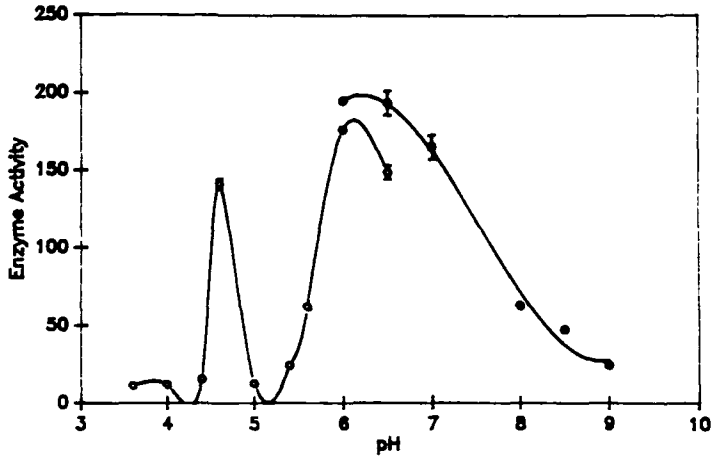


Fig. 1. Profile of acid phosphatase in whole cells as a function of pH. *T. denticola* 33520 was grown to early stationary phase, washed and assayed. Enzyme activity is expressed as nMol pNP released per mg soluble protein. Values represent average ± standard deviation of triplicate samples. Open symbols, 0.1 M sodium acetate buffers; filled symbols, 0.1 M Tris-HCl buffers.

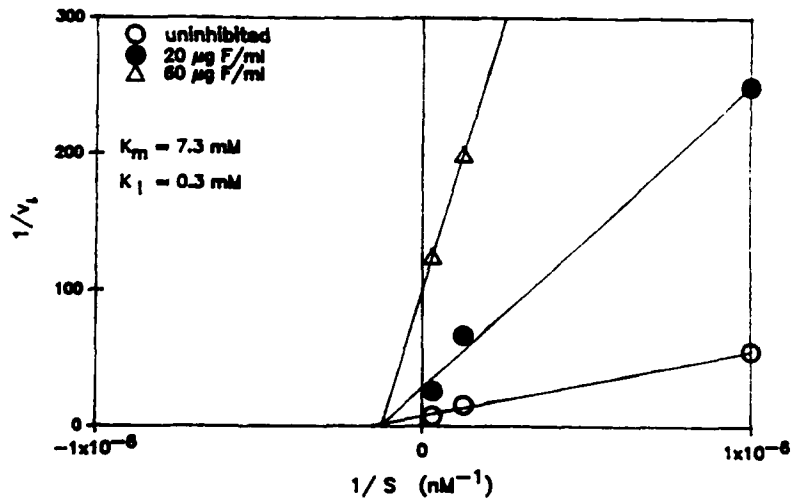


Fig. 2. Lineweaver-Burk plots and Michaelis constants of the acid phosphatase of intact cells of *T. denticola*. Assay mixtures contained early-stationary phase *T. denticola* 33520 and pNPP as substrate. The initial slope of a curve representing the release of pNP as a function of time was calculated to determine reaction velocity.

### *Michaelis constant for nonspecific acid phosphatase*

Kinetic parameters for the hydrolysis of pNPP by intact cells were determined by measuring the release of pNP at various times and concentrations. The initial slope of a curve which represents the release of pNP as a function of time was calculated to determine reaction velocity. The apparent $K_m$ and $K_i$ for sodium fluoride were determined by the method of *Lineweaver* and *Burk* (14). As shown in Fig. 2, fluoride appears

to be a noncompetitive inhibitor of the enzyme in intact cells, with an apparent $K_i$ of 0.3 mM, and apparent $K_m$ of 7.3 mM.

*Effect of fluoride on acid phosphatase in cell extracts of T. denticola*

In an effort to understand enzyme activity at a level beyond that of the intact cell, so that the acid phosphatase could be isolated and characterized, whole cells of *T. denticola* In an effort to understand enzyme activity at a level beyond that of the intact cell, so that the acid phosphate could be isolated and characterized, whole cells of *T. denticola* 33520 were disrupted with the French pressure cell, as outlined in Materials and Methods. The resulting cell extract was assayed for acid phosphatase in the presence and absence of fluoride (Table 1). Fluoride was added to the assay mixture at time 0. The addition of 20 and 40 μg/ml F– (as NaF) significantly decreased acid phosphatase in disrupted cell suspensions. The addition of 10 μg/ml $F^-$ (as $SnF_2$) induced a 38% decrease in the activity of acid phosphatase. Concentrations of $F^-$ higher than 10 μg/ml as $SnF_2$ could not be employed due to precipitation of the compound.

Table 1. Effect of fluoride on acid phosphatase in cell extracts of *T. denticola*

| Sample | nMols pNP released per mg soluble protein[a] |
|---|---|
| Cell extract[b] | 23.1 ± 5.4 |
| Cell extract, boiled 15 min | 1.5 + 0.6 |
| Cell extract + 5 μg $F^-$/ml ($SnF_2$) | 17.5 ± 8.2 |
| Cell extract + 10 μg $F^-$/ml ($SnF_2$) | 14.3 + 5.7 |
| Cell extract + 5 μg $F^-$/ml (NaF) | 23.5 ± 9.2 |
| Cell extract + 10 μg $F^-$/ml (NaF) | 18.9 ± 5.6 |
| Cell extract + 20 μg $F^-$/ml (NaF) | 11.7 + 2.3 |
| Cell extract + 40 μg $F^-$/ml (NaF) | 10.3 + 1.0 |

[a] Values reflect mean ± standard deviation of at least 3 separate experiments. Underlined values indicate a level of significance $P \leq 0.01$.

[b] Whole cell suspensions were passed through the French pressure cell twice at 16,000 lb/in$^2$, as previously described.

*Localization of acid phosphatase*

An analysis of the cell fractionation scheme revealed that the acid phosphatase was present in all subcellular fractions of *T. denticola* 33520 (data not shown). Although the acid phosphatase of *T. denticola* seemed to behave like the corresponding *E. coli* enzyme by exhibiting activity in intact cells (17), the activity did not appear to be efficiently released by osmotic shock procedures. This inefficient osmotic shock release is also characteristic of *S. typhimurium* phosphatases (11). When freshly prepared subcellular fractions were assayed for hexokinase activity, a cytoplasmically located enzyme, the osmotic shock fluid ("periplasmic contents") was not contaminated with hexokinase activity (data not shown). Therefore, the osmotic shock procedure did not

appear to damage the cytoplasmic membrane and result in the release proteins from the cytoplasmic contents.

Another approach to protein localization of periplasmic enzymes involves the use of reagents incapable of penetrating the bacterial permeability barrier, the cytoplasmic membrane (18). Enzymes inside the cytoplasmic membrane cannot be inactivated unless the cells are disrupted; enzymes on, or outside the membrane can be inactivated. With one such reagent, 7-diazonium-1,3-naphthalene disulfonate (diazo-NDS), it is possible to determine whether an enzyme lies inside the cell membrane, or whether it is exposed to the external environment. Whole cells and osmotically shocked cells were treated with this reagent. The shocked cells were then disrupted to determine the distribution of the enzyme in cell fractions. Based on color observations (not shown), the orange reagent, diazo-NDS, bound to the membrane fractions of the cells. Diazo-NDS inhibited acid phosphatase in whole cells and in shocked cells (Table 2). In diazo-NDS treated shocked cells that were further fractionated to "membrane" and soluble "cytoplasmic contents", the membrane-associated enzyme activity was inhibited, whereas the soluble activity was not. Therefore, diazo-NDS did not penetrate the cytoplasmic membrane of the cell. The increased activity in the diazo-NDS treated shock cell "cytoplasm" could not be explained. These results provide evidence that the acid phosphatase is exposed to the periplasmic space of the organism.

Table 2. Effect of 7-diazonium-1,3-naphathylene disulfonate (diazo-NDS) on acid phosphatase of *T. denticola*

| Treatment and Fraction[a] | nMol pNP released per mg soluble protein[b] |
|---|---|
| Untreated, whole cells | 24.0 ± 1.1 |
| Diazo-NDS, whole cells | 3.0 ± 2.4 |
| Untreated, shocked cells | 21.1 ± 1.4 |
| Diazo-NDS, shocked cells | 8.6 ± 3.6 |
| Untreated shocked, French press, "membrane" | 16.7 ± 0.01 |
| Diazo-NDS shocked, French press, "membrane" | 1.8 ± 0.8 |
| Untreated shocked, French press, "cytoplasm" | 18.2 ± 2.7 |
| Diazo-NDS shocked, French press, "cytoplasm" | 55.3 ± 9.2 |

[a] Diazotization and cell fractionation scheme as previously described.
[b] Activity measured by release of pNP as previously described. Values represent average ± standard deviation of triplicate samples.

## Discussion

In this study, acid phosphatase has been identified as a potential target for fluoride action in *T. denticola*. In intact cells, fluoride, as NaF, appears to be a classical non-competitive inhibitor of enzyme activity. Fluoride inhibits the growth of this organism (8). These studies do not suggest that the acid phosphatase is the only target for fluoride action. The potential effects of fluoride on membrane potential, extracellular and intracellular pH or transport mechanisms were not considered (1, 10, 12). Furthermore, other fluoride target molecules may exist in *T. denticola*.

Most of the properties of the *T. denticola* acid phosphatase measured in intact cells resemble those described for the corresponding *Salmonella* (11, 21) and *E. coli* enzymes (3, 5). Evidence has also been provided suggesting a periplasmic location for the acid phosphatase in *T. denticola*. Admittedly, evidence for the location of the enzyme in this study is indirect. The primary evidence for the periplasmic location of the *T. denticola* acid phosphatase is that the enzyme is partially released in osmotic shock fluids, and that the enzyme is able to hydrolyze phosphate esters in whole cells.

The methods used here to study the periplasmic location of the acid phosphatase are selective. Osmotic shock does not release any internal, cytoplasmic proteins. Another approach, utilizing the reagent diazo-NDS, inactivates proteins (enzymes) on, or outside the cytoplasmic membrane, while proteins inside the permeability barrier are unaffected unless the cells are disrupted. The charged sulfonate groups appear to prevent its penetration into the cell. A disadvantage to using diazo-NDS is that if a protein does not contain an adequate number of histidine, or tyrosine residues diazo-NDS will not be bound and inactivation will not occur (18). The amino acid content of the acid phosphatase of *T. denticola* is not presently known.

Although acid phosphatases have not specifically been implicated as virulence factors in periodontal disease, they may play an important role in the metabolism of *T. denticola* in the oral cavity. *T. denticola* cell-bound enzymes, if located on, or near the spirochetal cell surface, can hydrolyze substrates present in the periodontal pockets, or in the tissues if the spirochetes are invasive. This implies that the enzymes are always available to hydrolyze host components. In the case of a nonspecific acid phosphatase, the enzyme would be available to remove phosphate groups from host-produced nontransportable phosphate esters, thereby allowing the hydrolyzed host components to be transported and utilized by the cell. The phosphatase activity could supply a variety of nutrients to the cell depending on the ester's organic moiety, in additon to phosphate. Para-nitrophenyl phosphate has been used as a substitute for nonspecific phosphatases (11, 17). An understanding of the role of acid phosphatases in *T. denticola* appears tied to the fluoride-sensitivity of the organism.

A possible role for the periplasmically-exposed acid phosphatase of *T. denticola* would appear to be that of a scavenging enzyme. The presence of this periplasmic enzyme would enable the spirochete to utilize phosphate-containing compounds present in the growth environment. In the event of periodontal disease, this efficient use of nutrients within the periodontal pocket would allow the oral spirochete to compete and thrive.

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*Carrie A. Norton Hughes,* Ph.D., Department of Microbiology, University of Minnesota Medical School, Box 196, UMHC, 420 Delaware Street, S.E., Minneapolis, MN 55455, USA